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PHYSIOLOGICAL ACTIONS OF THE PARTIALLY PURIFIED SERUM VASOCONSTRICTOR (SEROTONIN)

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Rapport *et al.* (1948a) made a stable preparation of the vasoconstrictor of blood serum in the form of a nitro-barbiturate complex. After further purification the active substance was shown on chemical grounds to be an indole derivative—5 hydroxy tryptamine (Rapport, 1949). There appears to be only one very brief report about the pharmacological effects of the active substance which refers to its vasoconstrictor action in the rabbit's ear, its pressor action in the cat and its stimulating action on the rabbit ileum (Rapport *et al.* 1948b).

The purpose of this paper is to describe the pharmacological actions of the serum vasoconstrictor which we have prepared from beef serum by the method of Rapport *et al.* (1948a) and to discuss such actions in relation to previously published data about the effects of whole serum and of the indolalkylamines, tryptamine (Laidlaw, 1911; Reid, 1951) and bufotenin (Chen, Jensen and Chen, 1931; Erspamer, 1946). Evidence will thus be presented, on biological grounds, that the active substance is an indolalkylamine derivative.

METHODS.

Preparation from beef serum of the active substance as a nitro-barbiturate complex.

Blood was collected from cattle at the abattoirs (a) in five litre flasks, the serum being separated from the clot by centrifugation in a large Jouan centrifuge within a few hours of collection; or (b) in a galvanised iron can holding 30 to 40 litres and immediately defibrinated. After straining off clots through wire gauze the cells were separated from the defibrinated blood in a "Cepa Schnell Zentrifuge." By the latter method 10 litres of serum could be separated from the cells in half to one hour.

The serum was treated in the five stages described by Rapport *et al.* (1948a) in volumes ranging from 1·5 to 9 litres.

Stage 1. The pH was adjusted to 4·5 with 2N HCl, the proteins were precipitated with ethanol and the filtrate was concentrated below 40° C. in a climbing film evaporator. This apparatus obviated the use of nitrogen which Rapport *et al.* found necessary and greatly shortened the time necessary for concentration (30 to 60 minutes per litre of filtrate).

Stage 2. Acetone-insoluble impurities were precipitated from the concentrated filtrate by the addition of 10 volumes of acetone and mechanical stirring. After filtration the filtrate was again concentrated to a final volume 60 to 140 ml. in the film evaporator, the final traces of

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acetone being removed in a vacuum still. The concentrated filtrate was opalescent yellow or greenish yellow in colour and there was a greenish oil floating on top. Only once in eight runs was the oil dark brown in colour as described by Rapport.

Stage 3. The filtrate was extracted with an equal volume of chloroform. The aqueous layer was light yellow and the chloroform layer was yellow-green, or green in colour.

Stage 4. The aqueous layer was adjusted to pH 5·6, saturated with ammonium sulphate, extracted with butanol and the extract left overnight at —10° C.

Stage 5. The active substance was precipitated from the butanol extract by a solution of 5-nitrobarbituric acid in methanol.

The mass of the nitrobarbiturate complex so obtained varied in eight runs from 100 to 590 mg. per litre of serum (average 265 mg.). This complex is composed mainly of the ammonium salt of the precipitating agent and less than 1 p.c. of it is the active substance (Rapport, 1949).

The loss of activity was determined in one of two ways: 1. A sample of the original serum was frozen at —10° C. until the preparation had been completed when it was matched against the preparation on the carotid artery of the sheep or ox. 2. A sample of the serum freshly obtained was matched against the nitrobarbiturate complex from another batch and the final product was matched against this same standard. The loss of activity varied from about 50 to 80 p.c.

In order to be sure that the effects to be described were not due to the nitrobarbiturate, control solutions were used with every test object. These were (a) a solution of ammonium nitrobarbiturate or (b) a preparation made as already described but starting from pharmacologically inactive serum prepared from "platelet free" plasma (see later).

Active and control solutions were prepared in a concentration of approximately 3 mg. per ml. of 0·9 p.c. saline.

Biological test-objects.

Except where otherwise stated, cats were anaesthetized with chloralose after preliminary ether anaesthesia. The animals breathed naturally or were artificially ventilated. The systemic arterial blood pressure was recorded from the left carotid artery and the pressure in the right ventricle through a plastic catheter inserted via the right jugular vein. Sometimes, the chest was opened and the pressure recorded in a branch of the pulmonary artery. All these pressures were recorded with mercury manometers. A saline manometer connected to a Brodie tambour was used to record pressure in the pulmonary vein. The respiratory movements and intrapleural pressure were recorded as previously described (Reid, 1951).

Preparations were tested for their vasoconstrictor activity in the hind limb of the cat by the method of Feldberg and Schilf (1930) as described by Reid (1951). To test for vasoconstrictor activity in the kidney of the cat the animal was eviscerated, the aorta tied below the renal vessels, the adrenals removed and the outflow from the left renal vein measured and returned to the jugular vein. Injections were made into the cut stump of the coeliac artery.

The action on the suprarenal medulla of the eviscerated cat was observed by the method of Feldberg and Minz (1931), the aorta being ligated below the renal vessels which were also tied and injections were made into the cut stump of the coeliac or superior mesenteric artery. The contraction of the right nictitating membrane which was denervated by removal of the superior cervical ganglion 10 or more days previously was recorded together with that of the innervated nictitating membrane, and the blood pressure was recorded from an axillary artery.

The action on the nictitating membrane and pupil was observed in eviscerated adrenalectomised cats, injections being made intravenously or by means of a fine hypodermic needle into a carotid artery. The movements of the pupil were also observed in cats decerebrated under ether anaesthesia, care being taken to see that the third nerves had been divided. Both carotid arteries were left unligated and injections were made directly into them. The effect on the isolated cat's pupil was studied by the method used by Burn (1932).

The contraction of a horn of the uterus of the virgin cat *in vivo* was recorded on a smoked drum. The uterus of the virgin rat or guinea-pig, the jejunum of the guinea-pig and isolated spiral strips of carotid artery from sheep, dog or ox were suspended in 10 ml. isolated organ baths in Anaphylactic Ringer, Tyrode or Ringer solutions respectively.

RESULTS.

Circulatory effects in the cat.

The arterial pressures in the systemic and pulmonary circulations.

In the past it has not been possible to study the vascular effects of the serum vasoconstrictor in the whole animal because it was not possible to obtain, for injection, solutions containing the active substance in sufficient concentration. Now it is possible to inject, say, 24 mg. of nitrobarbiturate complex in a volume of 8 ml. In our most concentrated preparation such an amount was derived from 240 ml. of beef serum and with a 40 p.c. recovery, this would actually represent the activity in 96 ml. of serum.

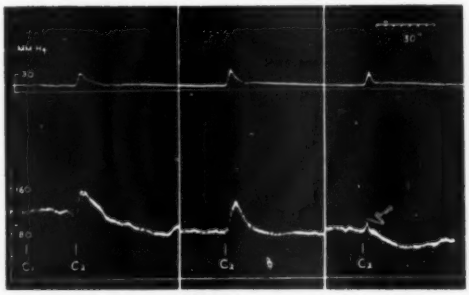


Fig. 1.

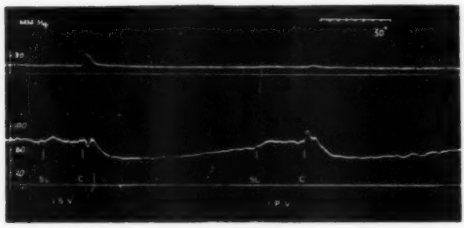


Fig. 2.

Fig. 1. Record of pressure in the right ventricle (upper trace) and pressure in carotid artery (lower trace) in a cat weighing 2·8 kg. under chloralose anaesthesia. At C_2 21 mg. of serum vasoconstrictor were injected intravenously. At C_1 a similar amount of a preparation made from serum prepared from platelet-free plasma was injected. Between the last two panels yohimbine (6 mg.) was injected.

Fig. 2. Record of pressure in pulmonary artery (upper trace) and pressure in carotid artery (lower trace) of a cat weighing 2·7 kg. under chloralose anaesthesia with open thorax. At C 16 mg. of the serum vasoconstrictor were injected intravenously at I.S.V. or into a pulmonary vein at I.P.V. Saline was injected at SL.

Rapport *et al.* (1948b) show a pressor action in the systemic circulation of the cat, but an inspection of their Figure suggests that successive injections were given without waiting for the completion of the action of each preceding one. We have found the action of serum vasoconstrictor on the arterial blood pressure to be variable. Sometimes a shortlived simple pressor response is obtained as in Figs. 1, 3 and 7; sometimes the rise of blood pressure is preceded by a fall as in Figs. 2 and 6; sometimes the rise of blood pressure even with a large dose is negligible as in Fig. 2; and sometimes the rise of pressure is followed by a prolonged fall (Figs. 2 and 5). It is interesting to note that all these variations were seen in the effects of tryptamine (Reid, 1951).

Following the intravenous injection of serum vasoconstrictor the pressure in the right ventricle or in the pulmonary artery regularly rises, followed in a few seconds by a rise of pres-

sure in the carotid artery. During the rise of pressure in the pulmonary artery the pressure in the pulmonary vein falls and sometimes the systemic arterial pressure falls initially at the same time. Fig. 1 shows the records of the pressure in the right ventricle and the pressure in the carotid artery of a cat weighing 2·8 kg. with divided vagi under artificial respiration. At C_2 21 mg. of a nitrobarbiturate complex representing the activity in about 80 ml. of beef serum was injected. Before the last injection, 6 mg. of yohimbine were given. The effect has been to reduce the pressor response in the lesser circulation and almost abolish the rise of pressure in the greater, bringing to light a somewhat prolonged depressor effect. The first injection given at C_1 is 24 mg. of a nitrobarbiturate complex prepared from pharmacologically inactive serum made from platelet-free plasma.

The rise of pressure in the right ventricle is due to increased resistance in the lungs, and not due to cardiac stimulation, for it is not obtained when injections are made directly into the pulmonary vein. Fig. 2 shows the pressure in the pulmonary and carotid arteries in a cat weighing 2·7 kg. under chloralose anaesthesia and artificial ventilation with vagi divided. At C 16 mg. of a nitrobarbiturate complex were injected. The first injection was made into a saphenous vein. It caused a rise of pressure in the pulmonary artery; the systemic arterial pressure fell initially, rose slightly and then gave a prolonged fall. The second injection which was given into a pulmonary vein caused no rise of pulmonary arterial pressure, no initial systemic depression and a greater rise of systemic arterial pressure than previously which was again succeeded by a prolonged depression. Control injections of physiological saline were given at SL.

Vasoconstriction in the hind limb.

Fig. 3 shows the blood pressure and outflow from the femoral vein in a cat weighing 3 kg. under artificial ventilation with divided vagi. Except when indicated by the letters I.V. the injections were made through the cut stump of a muscular branch of the femoral artery. At SR 0·5 ml. and 1·0 ml. respectively of this cat's own serum was given. At the three marks labelled C_1 0·5, 1·5 and 3·0 mg. respectively of a preparation of the vasoconstrictor were injected, 1 mg. representing the activity in approximately 1·5 ml. of the original beef serum. All these injections caused vasoconstriction as evidenced by the diminished outflow. At C_2 14 mg. of another preparation were given intravenously; this represented about 10 ml. of the original serum. It caused a rise of blood pressure together with vasoconstriction in the hind limb. For comparison the effect of 0·2 mg. tryptamine injected intra-arterially is shown at T. Control injections of physiological saline were given at S. and were without significant action.

Sometimes with repeated injections of the vasoconstrictor the response declines. It is also reduced in magnitude by the intravenous injection of yohimbine.

Vasoconstriction in the kidney.

Fig. 4 shows the arterial blood pressure and the outflow from the left renal vein of an eviscerated adrenalectomised cat with divided vagi under artificial ventilation. Injections were made into the cut stump of the coeliac artery, the aorta being tied below the level of the renal vessels. At SR 1 and 3 ml. respectively of the cat's own serum were injected causing as can be seen, a diminution in blood flow with a slight rise of blood pressure. Physiological saline was injected at SL, the larger dose of 3 ml. causing an increased outflow presumably due to a volume effect. At C 9 mg. of serum vasoconstrictor were injected, this mass being contained in a volume of 3 ml. and representing the activity of about 6 ml. of beef serum. The injection of 5 μg. of histamine hydrochloride in 0·5 ml. caused an increase in outflow and finally 1 mg. of tryptamine caused a slight pressor response together with a diminution in outflow. Our past experience (Reid, 1951) has been that the action of tryptamine in the kidney is weak compared with its effect in the hind limb and the response recorded here was unexpectedly great.

Respiratory effects in the cat.

The intravenous injection of serum vasoconstrictor in the cat causes broncho-constriction as evidenced by an increase in volume of the chest and diminished respiratory movements together with an increase in the range of variation of intrapleural pressure which continues for some minutes after the depth of ventilation has returned to normal. In addition to the bronchoconstriction there may be an initial apnoeic pause lasting for one or two respiratory cycles. Similar effects have already been described for tryptamine (Reid, 1951).

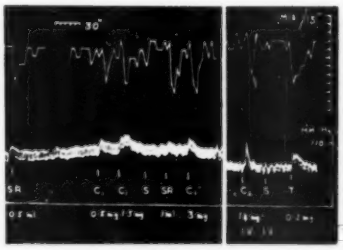
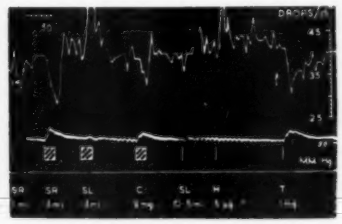


Fig. 3.



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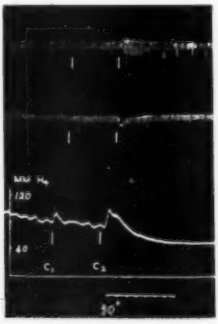


Fig. 5.

Fig. 3. Records from a cat weighing 3 kg. under chloralose anaesthesia, and artificial respiration with vagi cut. The upper record is the outflow from the femoral vein traced after varnishing, the lower is the carotid blood pressure. Serum vasoconstrictor is denoted by C_1 or C_2, serum prepared from this cat by SR, saline by S, and tryptamine by T. Except for the first two injections in the second panel, injections were made into a branch of the femoral artery.

Fig. 4. Records from an eviscerated cat weighing 4·4 kg. under chloralose and artificial respiration with vagi cut, the upper record being the outflow from the left renal vein traced after varnishing, and the lower one the carotid blood pressure. Injections were made into the coeliac artery. SR denotes cat serum; SL, saline; C, serum vasoconstrictor; H, histamine, and T tryptamine.

Fig. 5. Records (from above down) of intrapleural pressure, respiratory movements and blood pressure in a cat weighing 3 kg. under chloralose anaesthesia. Lettering as in Fig. 1. Details in text.

Figs. 5 and 6 illustrate the results obtained. In each Figure, the records from above down are of intrapleural pressure, respiratory excursions recorded with a stethograph and the arterial blood pressure. A downward movement denotes inspiration. At C_1 a control injection of a nitrobarbiturate complex made from inactive serum (prepared by clotting platelet-free plasma) was injected and at C_2, 20 mg. of serum vasoconstrictor were given intravenously. In Fig. 5, a very brief period of apnoea precedes the bronchoconstriction. In Fig. 6, initially there is a transient increase in respiratory rate and thereafter the respiratory excursion as well as being reduced in amplitude is irregular in rate and depth.

Action on the suprarenal gland.

In an earlier paper it was shown that tryptamine is without direct action on the suprarenal medulla, and it was suggested that the substance in serum responsible for the liberation of adrenaline must be other than the one isolated by Rapport unless that particular tryptamine

derivative possessed such an action not shared by tryptamine itself. We now find that the partially purified serum vasoconstrictor stimulates the adrenal medulla when injected into the cut stump of the superior mesenteric artery in eviscerated animals. Fig. 7 illustrates such an experiment. It shows records of the behaviour of both pupils, both nictitating membranes and the blood pressure in an axillary artery of a cat weighing 5 kg. in which the right superior cervical ganglion had been removed 11 days beforehand. Injections were given intra-arterially or intravenously as denoted by the letters I.A. and I.V. respectively. Adrenaline was given at A, saline at S and serum constrictor at C. The intra-arterial injection of the latter caused the liberation of active substances from the adrenal medulla as indicated by the rise of blood pressure, increase of heart rate, dilatation of the pupil and contraction of the nictitating membranes, the latter two responses being more marked on the denervated side. The intravenous injection of the serum vasoconstrictor in the same dose was without pressor action, the pupil constricted slightly in place of the dilatation shown previously and the nictitating membrane contracted only slightly. It will be shown presently that these responses of pupil and nictitating membrane are due to a direct action of the serum vasoconstrictor on these tissues.

Action on the nictitating membrane and pupil.

Between the two panels of Fig. 7 the suprarenal glands were removed. The intravenous injection of 7·5 mg. of vasoconstrictor now caused a pressor response together with contraction of the nictitating membranes and constriction of the pupils which lasted about as long as the contraction of the denervated nictitating membrane. The effect of the intravenous injection of 1·0 mg. of tryptamine is shown for comparison. It should be noted that the response of the nictitating membrane to both tryptamine and to the vasoconstrictor is greater on the denervated side. This has already been demonstrated for tryptamine and for cat serum (Reid, 1951). Sometimes the pupillary constriction due either to tryptamine or the vasoconstrictor is preceded by a transient dilatation.

We occasionally find that denervation of the nictitating membrane does not potentiate its response to tryptamine, an observation which recalls those made by Bulbring and Burn (1938) with tyramine. When the effect of tryptamine is not potentiated, neither is the effect of serum vasoconstrictor. Fig. 8 illustrates such an experiment. The records are of the denervated and normal nictitating membranes and pupils of an adrenalectomised cat under chloralose anaesthesia. The right nictitating membrane has been sensitised to 2 μg. of adrenaline (at A) but not to 12 mg. of serum vasoconstrictor (at C) nor to 1 mg. of tryptamine (at T). At the arrow a control injection of ammonium nitrobarbiturate was given.

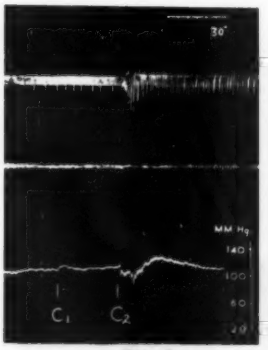
The site of action on the pupil of both serum vasoconstrictor and tryptamine has been investigated in low section decerebrate cats in which the third cranial nerves were divided; the carotid arteries were left unligated. The injection into a common carotid artery of either tryptamine or vasoconstrictor causes constriction of the pupil which is obtained whether or not the sympathetic has been divided or the animal given atropin. These results indicate that the action of these agents is directly on the smooth muscle of the pupillary sphincter. Histamine hydrochloride in doses ranging from 1·5 to 1,000 μg. causes dilatation.

The isolated pupil of the cat in a 5 ml. bath of Ringer solution constricts on the addition of 1 or more mg. of vasoconstrictor. Histamine in doses greater than 2 μg. causes dilatation.

Action on the isolated ring or spiral strip of artery.

As already indicated under Methods, these test objects were used to determine the loss of activity of the various preparations. The vasoconstrictor stimulated arterial strips of ox, dog or sheep after a latency of 5 to 20 seconds. The sheep carotid was a more satisfactory test object than the ox carotid; it relaxed more readily and remained a satisfactory preparation for two or three hours. Fig. 9 shows the type of response obtained. In the first part of the Fig.,

the unlettered responses are due to 0·4 mg. of serum vasoconstrictor; 5 μg of histamine chloride were without effect at H. The drugs were left in contact for 60 secs., before washing out. At C 0·4 mg. of serum vasoconstrictor was left in contact for a prolonged period during which 0·5 mg. of yohimbine relaxed the artery which became unresponsive to the constrictor for a period of 45 minutes and still gave reduced responses after 1 hour. Similar antagonism between yohimbine and whole serum and between yohimbine and tryptamine has been recorded before (Reid, 1946, 1951).



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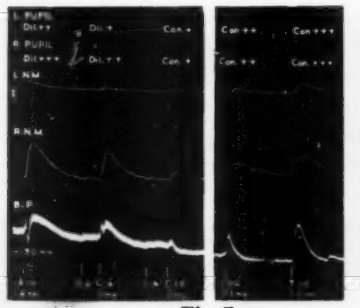

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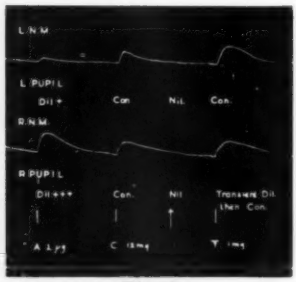

Fig. 8.

Fig. 6. Records as in Fig. 5. At C_2 20 mg. of serum vasoconstrictor were injected intravenously.

Fig. 7. Records from an eviscerated cat weighing 5 kg. under artificial respiration and chloralose anaesthesia with vagi cut. Records from above down are L pupil, R (denervated) pupil, left nictitating membrane, right (denervated) nictitating membrane and pressure in an axillary artery. Adrenaline was injected at A, saline at S, vasoconstrictor at C, and tryptamine at T. The letters IV and IA denote intravenous injection or injection into the cut stump of the superior mesenteric artery respectively. Between the two panels adrenalectomy was performed.

Fig. 8. Records from an eviscerated adrenalectomised cat under chloralose anaesthesia and artificial respiration. Records from above down are of left nictitating membrane, left pupil, right (denervated), nictitating membrane and right pupil. Injections were made intravenously, being denoted by letters as in Fig. 7. At the arrow ammonium nitrobarbiturate (12 mg.) was injected.

In general, the experiments with the sheep arterial strips show that while histamine itself is without action, the subsequent response to vasoconstrictor may be unaffected, potentiated or depressed. When added during a sustained contraction, histamine causes a further contraction followed by relaxation. The second part of Fig. 9 shows the response of another piece of sheep artery to 0·4 mg. of serum vasoconstrictor. This tracing shows how 10 μg. histamine while without effect alone, caused at first a contraction followed by relaxation when added during a sustained contraction, the artery returning to its state of contraction when the bath was washed out and the constrictor re-added.

Other test-objects.

The rat uterus. The responses of the rat uterus to this preparation are similar to those already described for serum itself and for tryptamine. Although responses to successive doses decline, this test-object can be used to compare the activities of different samples (cf. Erspamer, 1946).

The uterus of the guinea-pig. This test object contracts on the addition of serum vasoconstrictor after a latency of several seconds. On washing out relaxation is slow. Like the rat uterus it declines in sensitivity, but unlike it, it is an unsatisfactory preparation for assay purposes, a conclusion which Erspamer (1946) also reached in his work on bufotenin.

The jejunum of the guinea-pig. The guinea-pig jejunum responds to the serum vasoconstrictor after a latency of a few seconds and its response is similar to that given by untreated histamine-free serum. When it is left in contact, the contraction is not sustained, and if histamine is added to the bath before washing out, there is a good response to it followed after washing out by a period of depression lasting several minutes. When doses of constrictor are repeated at intervals of 10 minutes or more during which histamine responses are elicited (cf. Fig. 12) roughly quantitative comparisons between samples may be made. When injections are repeated at intervals of a minute or so the responses rapidly decline in magnitude, as do also the responses to tryptamine. Fig. 10 illustrates this tachyphylaxis to 0·3 mg. of the serum vasoconstrictor at C and Fig. 11 shows the similar tachyphylaxis to 1·4 mg. of tryptamine at T. It can be seen that repeated doses of tryptamine or constrictor depress temporarily the response to histamine and Fig. 11 shows that when the gut has become refractory to tryptamine it is also refractory to the vasoconstrictor.

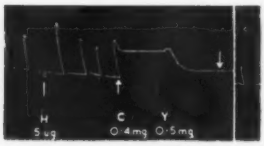

Fig. 9.

Fig. 10.

Fig. 9. Records of contraction of two strips of carotid artery of the sheep. In the first panel of the Figure the unlettered contractions are due to 0·5 mg. of serum vasoconstrictor and 5 µg. of histamine were injected at H. At C, 0·4 µg. of serum vasoconstrictor was injected and left in contact during which 0·5 mg. of yohimbine was injected at Y. The bath was washed out at ↓. Interval between panels 1 hour. In the second half of the Figure, 10 µg. of histamine were given at H during the sustained contraction due to 0·4 mg. of constrictor (at C). At the third arrow the bath was washed out, and 0·4 mg. of constrictor was added, the bath being again washed at the fourth arrow. The last contraction (at C) is to 0·4 mg. of serum vasoconstrictor.

Fig. 10. Record of guinea-pig jejunum. The unlettered contractions are due to 0·1 µg. of histamine. 0·25 mg. of serum vasoconstrictor was injected at C. Interval between panels 5 minutes.

The response of the gut to the vasoconstrictor of serum or to tryptamine is antagonised by benadryl, but as the gut recovers from its action it can be seen that the antagonism towards these agents is not quite so great as it is toward histamine.

The blood vessels of the human skin. A concentrated solution of our most potent preparation (the one used in the experiments of Figs. 1, 2 and 5) was without blanching action when injected intradermally.

The source of the vasoconstrictor in serum.

Is the substance which has been obtained in the nitrobarbiturate complex that which is responsible for the vasoconstrictor activity of the original serum or is it an artefact? That something which is present in the original serum has been isolated is indicated by the fact that

its actions on a great variety of test objects are shared by serum itself. Rapport *et al.* (1948a) followed the vasoconstrictor activity through the various stages from serum itself to the final product, and the results indicated that the vasoconstrictor substance of serum had in fact been isolated.

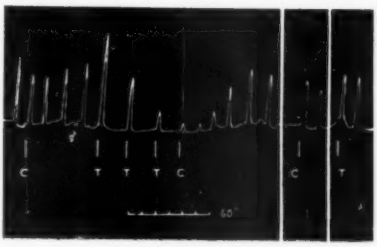

Fig. 11.

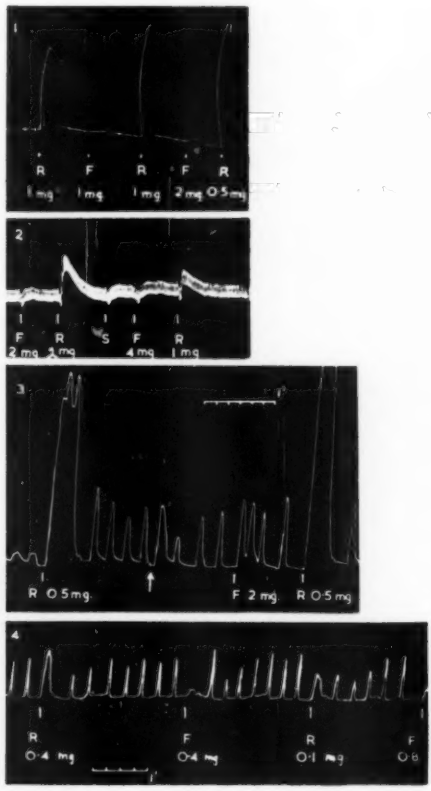

Fig. 12.

Fig. 11. Record of guinea-pig jejunum. The unlettered contractions are due to 0·2 μg. of histamine; 0·3 mg. of serum vasoconstrictor was injected at C and 1·4 mg. of tryptamine at T. Interval between panels, 5 minutes and 2 minutes respectively.

Fig. 12. Records from above down are of the ox carotid artery, the blood pressure of an eviscerated cat, the uterus of virgin rat and jejunum of the guinea-pig respectively. At R a nitrobarbiturate complex prepared from serum made from "platelet-rich" plasma was injected into the bath or (on panel No. 2), into the cut stump of the coeliac artery. At F the nitrobarbiturate complex prepared from the serum made from corresponding "platelet-free" plasma was injected. The figures denote milligrammes. At S in panel 2, 1·0 ml. of saline, and at the arrow in panel 3, 2 mg. of ammonium nitrobarbiturate were injected.

Now it has been shown elsewhere (Reid and Bick, 1942) that the activity of serum depends on the presence of platelets in the plasma or blood at the time of clotting. Therefore if we prepare serum by recalcifying highly centrifuged plasma, the nitrobarbiturate complex which it yields should be free or almost free from activity. Such has been found to be the case.

Blood was collected into silicone coated vessels using sodium citrate as an anticoagulant. It was spun lightly in a large Jouan centrifuge (500 *g.* at tip; 340 *g.* at surface) to throw down the bulk of the red and some of the white cells; the plasma was removed, pooled and respun in a refrigerated International centrifuge (Conical Head No. 845, 650 *g.* at tip). The resultant plasma (platelet-rich plasma) was opalescent and microscopic examination showed that its cellular content was composed almost entirely of platelets. A portion of this plasma was spun a third time (2,000 *g.* at tip) to throw down the suspended elements and obtain a clear "platelet-free" plasma. The sediment from the third centrifugation was added to the platelet-rich plasma. By these procedures two plasmas were obtained from the same sample of blood—one clear and cell free, the other opalescent and rich in platelets. Each was clotted by the addition of a solution of $CaCl_2$ and equal volumes of the sera so obtained (1 to 2 litres) were extracted in parallel in the five stages already described. The results were clear cut. Sera made from platelet-free plasma yielded complexes which were almost devoid of activity, not only on the arterial strip but also on the adrenal of the cat, the nictitating membrane, the pupil, the rat uterus, the guinea-pig uterus and the guinea-pig intestine. Sera made from the corresponding platelet-rich plasma always yield complexes which were active on all of the above-mentioned preparations. As has been illustrated in Figs. 1, 5 and 6, the platelet-free preparations were also inactive on the cat's vascular and respiratory systems.

Fig. 12 shows the responses due to two such paired preparations, one derived ultimately from platelet-rich plasma being denoted by R, and one derived from platelet-free plasma being denoted by F. From above down, the responses indicate the contraction of the ox carotid artery, the liberation of adrenaline from the suprarenal gland, the contraction of the rat uterus and the contraction of the guinea-pig jejunum (the unlettered responses being due to 0·1 μg. of histamine).

Is the active substance single or multiple?

The results, so far, indicate that a preparation can be made from serum which has an action on a great variety of biological test objects and that all these actions have the same source, being present or absent concomitantly with the presence or absence of platelets at the time of clotting. Are all these actions due to one and the same substance?

The uniformity of behaviour of the nitrobarbiturate complex from various samples of serum suggests that one substance is responsible. The activity of our various samples was tested on a sheep carotid strip using one of them as a temporary standard. Estimates were made by a method of bridging or by a statistical method. The strongest was about five times as active as the weakest. The ratio between the activities could not be assessed so accurately on the other test objects, but approximate estimates were made by the method of bridging. In no case have we found a discrepancy between the activities of the different preparations when tested on the carotid artery, the guinea-pig jejunum, the rat uterus, the adrenal gland, the nictitating membrane and the pupil, which would suggest that we are dealing with more than one active substance in the complex.

Work is in progress to separate out from the complex the crystalline vasoconstrictor or serotonin. While so far we have not succeeded in doing so (having encountered some of the difficulties referred to by Rapport *et al.*, 1948c, in their less straightforward experiments) we have, as a result of our efforts, had available for testing, the various stages passed through in the course of the

fractionation process. As one would expect there is considerable difference between the activity of the various fractions (tested on the arterial strip). Irrespective of the particular test object, be it artery, gut, uterus, nictitating membrane or adrenal gland, the activity is distributed between the different fractions in ratios of the same order. Nor do the actions of these fractions on the other biological preparations ever suggest that we have separated out one kind of activity from another.

DISCUSSION.

In the past the multiplicity of the actions of serum has suggested a multiplicity of active substances. Thus Daly (1938) refers to both vasotonins and bronchotonins in defibrinated blood causing vasoconstriction and bronchoconstriction respectively. Histamine is known to occur in serum particularly that of certain species such as the rabbit; and this substance may be responsible when present in sufficient concentration for some of the muscle stimulating actions; but it cannot be responsible for the action on the rat uterus, its vasoconstrictor and pressor action in the cat nor for its action on the isolated arteries. Adenyl compounds also may be present in serum (Zipf, 1931), and when present may be responsible for its stimulating action on the uterus, but not for its vascular effects. A protein-like substance present in serum can cause pulmonary vasoconstriction and reflex cardiac and respiratory disturbances in the cat (Brodie, 1900).

It is clear then that there may be a number of active substances in serum. However, previous work (Reid, 1946; 1948) has shown that serum which is virtually histamine-free can stimulate the guinea-pig intestine; serum which is free from adenyl compounds can stimulate the rat uterus. Our present results show that the partially purified serum vasoconstrictor possesses not only vasoconstrictor properties but stimulates the cat's pupillary sphincter, the cat's nictitating membrane, the cat's adrenal medulla, the isolated rat uterus, the isolated guinea-pig intestine and causes bronchoconstriction and respiratory arrhythmia in the cat; and the evidence indicates that a single substance is responsible. Quite obviously this does not exclude the presence of other pharmacodynamic agents in serum, some of which as pointed out above are quite well known, and Zucker (1944) provides evidence for more than one muscle stimulating agent in platelet extracts; what it does do is to indicate that there is one particular substance which possesses all the properties which we describe. This is supported by the striking resemblance between the actions of our partially purified preparation and those of one group of substances, viz:—the indolalkylamines, together with the chemical identification by Rapport (1949) of the vasoconstrictor as a tryptamine derivative.

Until the purified or partially purified preparations were available, comparison of the pharmacological activities of serum with those of any known group of substances was unprofitable because of the possible multiplicity of

active agents in serum. Now it can be seen that the vasoconstrictor of serum resembles sympathomimetic amines in some respects and differs from them in others. Like the sympathomimetic amines it is pressor and vasoconstrictor, these actions being antagonised by yohimbine; it contracts the nictitating membrane, this action being potentiated by denervation or by cocaine and antagonised by yohimbine and it relaxes the virgin cat uterus *in situ*. Unlike the sympathomimetic amines it contracts the isolated rabbit intestine (O'Connor, 1912), it constricts the pupil, it causes bronchoconstriction and it contracts the guinea-pig intestine and the rat uterus.

Similarly the vasoconstrictor is like histamine in some respects, unlike it in others. Like histamine it stimulates the guinea-pig ileum being antagonised by benadryl nearly as well as is histamine, it stimulates the guinea-pig uterus, it causes bronchoconstriction and it stimulates the suprarenal medulla (not antagonised by benadryl). Unlike histamine are its vascular effects, its constricting action on the pupil of the cat, its action on the rat uterus, the tachyphylaxis seen in its action on the guinea-pig intestine and the longer latency of response of that tissue.

On the other hand the resemblances between this substance and indolalkylamines are very striking indeed, and may be summarized as follows:

1. Vasoconstriction in the cat's hind limb is given by both tryptamine and serum vasoconstrictor and the action of each is antagonised by yohimbine.

2. A systemic pressor action in the cat is caused by the vasoconstrictor, tryptamine (Laidlaw, 1911; Reid, 1951) and bufotenin (Chen *et al.*, 1931) and again the response which is similar in type with all three agents is antagonised by yohimbine.

3. The systemic pressor response is sometimes preceded or followed by a depression. This has been described in the cat, dog, and rabbit for tryptamine (Reid, 1951) and in the rabbit for bufotenin (Handowsky, 1920).

4. A rise of pressure in the pulmonary artery is caused by both serum vasoconstrictor and tryptamine (Reid, 1951) and the action of each is reduced by yohimbine.

5. Serum vasoconstrictor, tryptamine (Laidlaw, 1911; Reid, 1951) and bufotenin (Handowsky, 1920) cause a period of apnoea on intravenous injection in the cat. Tryptamine and vasoconstrictor cause bronchoconstriction.

6. Contraction of isolated arterial rings is given by tryptamine (Reid, 1951) and bufotenin (Handowsky, 1920) as well as by the substance in serum. In each case the contraction is well sustained and (for tryptamine and serum vasoconstrictor) is antagonised by yohimbine.

7. On the guinea-pig intestine, tryptamine, serum vasoconstrictor and bufotenin (Erspamer, 1946) cause contraction after a brief latency. The contraction is not sustained and with each agent repeated injections at short intervals cause diminishing responses. In this respect tryptamine and serum

vasoconstrictor show cross desensitization one to the other. Their actions are also antagonised by benadryl.

8. The rat uterus gives similar responses with serum vasoconstrictor, tryptamine (Reid, 1951) and bufotenin (Erspamer, 1946). The description of the actions of bufotenin given by Erspamer might have been written about the effects of serum.

9. The uterus of the virgin cat *in situ* is relatively insensitive to both tryptamine and serum vasoconstrictor; when it does respond it does so by relaxation.

10. The nictitating membrane of the cat is contracted by both tryptamine and serum. The response in each case is potentiated by denervation and by cocaine and antagonised by yohimbine.

11. The pupil of the cat is constricted by serum vasoconstrictor by tryptamine and by bufotenin (Handowsky, 1920; Chen *et al.*, 1931). This response is still obtained after atropinisation. This is an important property in identification for, apart from choline esters and anticholinesterases, substances which have this effect are rare.

There is thus only one outstanding qualitative difference between tryptamine and the serum vasoconstrictor—the former does not stimulate the suprarenal medulla. There is no difficulty in believing that a tryptamine derivative may have such an action. Raymond-Hamet (1942) states that bufotenin (which is N dimethyl 5 hydroxy tryptamine) exerts its pressor action in this way but unfortunately he gives no details of experiments in which he attempted to show that it acted directly on the adrenals.

It remains now to consider the physiological significance of some of the reactions here reported. Some of them, as for example the respiratory effects in the cat, required large doses. If beef serum is of the same order of activity as cat serum it would require an amount of active substance present in the greater part of the cat's blood volume to cause the effects shown in the Figures. This action while it has provided a point of similarity with indolalkylamines is unlikely therefore to be a physiological effect. It may possibly become important in such conditions as massive pulmonary embolism or in conditions in which there is a rapid disappearance of blood platelets as in anaphylaxis.

Rapport *et al.* (1948b) found that the pure substance, serotonin, was more actively vasoconstrictor, mass for mass, on the rabbit's ear than adrenaline. We have found that amounts of the nitrobarbiturate complex corresponding to a fraction of a ml. of serum constrict the vessels of the hind limb of the cat. Nevertheless, our experiments on the entire cat do not suggest that it is a stronger pressor agent than is adrenaline. Even the Figure in the paper of Rapport *et al.* (1948b) shows a very modest rise of arterial blood pressure with as much as 25 μg. of crystalline serotonin.

One reason why the pressor action is not well marked with small doses may

be thought to be due to the fact that it is destroyed in the lungs by enzymatic action. That this is not the entire explanation is illustrated by the experiment of Fig. 2, in which a slight pressor effect was obtained even when injections were made into a pulmonary vein. Another possible factor may be its apparent lack of action on the capillaries. In the intact animal other factors may modify the effects of systemic vasoconstriction. One of these is the constriction in the pulmonary bed. Another is the prolonged depressor action seen in Figs. 2 and 5. The nature of this depression has not been elucidated. With such large doses a central effect cannot be excluded. It is unlikely that a large scale and rapid liberation of active substance comparable to an intravenous injection occurs in the body as a physiological event. If it plays a part in the maintenance of arterial pressure as has been suggested by Mooltan *et al.* (1949) it is more likely to be by a widespread local constriction of vessels. This would be in keeping with its possible physiological rôle as a local constricting agent in haemostasis. Here it will reach its highest concentration locally at the seat of liberation and here its action is likely to be found. By contrast, adrenaline is liberated not only locally at its site of action but also from the suprarenal gland for general distribution in the blood stream.

SUMMARY.

The vasoconstrictor of serum (serotonin) has been prepared in a stable, partially purified form as a nitrobarbiturate complex by the method of Rapport *et al.* (1948a).

It has been shown that serum prepared from highly centrifuged ("platelet-free") plasma yields a preparation almost devoid of activity.

The vasoconstrictor causes a rise of pressure in the pulmonary artery of the cat and a rise of systemic arterial pressure which may be preceded or followed by a depression. It constricts the vessels of the hind limb and kidney of the cat and contracts the isolated artery of the sheep, dog or ox. The vascular actions are antagonized by yohimbine.

It causes contraction of the guinea-pig jejunum and uterus, the rat uterus, the nictitating membrane and pupillary sphincter of the cat, and relaxes the virgin cat uterus *in vivo*.

It liberates adrenaline from the adrenal medulla of the cat.

It causes bronchoconstriction and respiratory arrhythmia in the cat.

The evidence indicates that all these actions are due to a single substance and all of them except the action on the adrenal gland are mimicked by the indolalkylamine—tryptamine.

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AN ATTEMPT AT THE CHEMICAL IDENTIFICATION OF "NEUROCLETIN" (THE SUBSTANCE EVOKING AXON-SPROUTING)

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In previous experiments (Hoffman 1950) it was found that following partial denervation of the muscles of rats, intact axons sprouted new processes which re-innervated the muscle fibres. It was further demonstrated that injection of an ether extract of nervous tissue, or egg-yolk, into the muscles of normal rats evoked the outgrowth of new processes from motor-end-plates and nerve fibres, which wandered at random in the endomysial spaces. It was therefore suggested that the sprouting which occurs in partially denervated muscles is evoked by the diffusion of a lipid substance from the degenerating nerve-fibres, which stimulates surviving axons to sprout. Similar suggestions in regard to such phenomena have been made by Weiss and Edds (1945) and Weddell, Guttmann and Gutman (1941).

It was felt desirable to investigate further the nature of the nerve-sprouting ("neurocletic") stimulant, since this might throw some light on the mechanism of its action. Further impetus was provided by the suggestion of Edds (1951) as to the possible significance of nerve sprouting in the recovery of poliomyelitic muscles. The experimental results presented below are concerned with attempts to purify "neurocletic" extracts, and with studies of the active fractions obtained.

Biological Materials and Methods.

After stripping off the dura- and pia-mater, ox spinal cords were chopped into small pieces, and forced through a 1 mm. mesh. In large-scale preparations up to one hundred cords were used. In later experiments batches of sixty egg-yolks were homogenised in a Waring blendor.

Extraction of active material.

The minced spinal cords were placed in a large Erlenmeyer flask with approximately four volumes of ether, shaken for six hours, and allowed to settle overnight. The supernatant was then decanted and filtered, and the residue extracted again with two volumes of ether. Homogenised egg-yolk was similarly extracted. During hot weather all extractions were carried out in the cold-room at approximately + 4° C.

Biological tests.

2 ml. aliquots of a petroleum ether solution of the substance to be tested were evaporated in a stream of nitrogen, the dry solid (usually about 3—10 mg.) emulsified in about 0·5 ml. of the propylene glycol (which had previously been shown to be inactive) and injected directly into the *tibialis anterior* muscles of *normal* rats. Usually about 0·25 ml. was injected into each muscle. During injection, the needle was directed parallel to the long axis of the muscle, just below its surface, and immediately after injection the liquid was massaged throughout the muscle, to prevent local pressure damage. In each test at least two rats were used, a total of 120 rats having been used in these experiments. Two to four days after injection the animals were killed, and the injected muscles prepared for histological examination by the Loewit gold method, then examined for neurocletic response. The appearance of blindly ending ultraterminal processes of end-plates, or—less frequently—fine, non-myelinated freely-ending branches of nerve-fibres was taken as evidence of activity. In these experiments muscles were injected only once, in contrast to experiments described previously in which three or more injections were made at twenty-four hour intervals. It was therefore interesting to note that the outgrowing processes observed here were consistently shorter than those obtained after repeated injections. This may indicate a fairly rapid absorption of the extracts from their site of action. However, attempts to correlate the extent of experimental change with concentration of active substance, by means of serial dilution tests, have proved unsuccessful, and at present only qualitative information has been obtained. Marked variation in the extent of response to identical extracts has also been observed. Any appearance of change in the tissue of experimental animals has been accepted as a positive result; in effect, therefore, the test is "all-or-none."

Chemical Methods and Results.

Solvents. In all cases organic solvents were purified by standard methods.

Acetone fractionation.

The combined ether extracts were evaporated *in vacuo* below 25° C. The residue formed a semi-liquid mass containing water and some protein, apart from lipids. Repeated extraction with four volumes of acetone yielded a cloudy solution, which, on centrifuging, separated into a precipitate and two liquid phases. The lower phase, apparently immiscible with the upper acetone phase was rejected after several acetone washings.

Both acetone-soluble fraction and residual acetone-insoluble fraction were tested for activity; only the former gave a positive result.

Saponification.

The cold saponification technique was adopted, since hot saponification was found to inactivate the material. A concentrated petroleum ether solution of the acetone-soluble fraction was mixed with a calculated excess of 3 p.c. alcoholic sodium ethoxide, and the reaction allowed to proceed overnight at room temperature. Following this, equal volumes of water and petroleum ether were added to the gel, which then dissolved. The two phases were separated, and the petroleum ether phase thoroughly extracted with N/10 sodium hydroxide. Emulsions were encountered at this stage, and were generally overcome by leaving overnight in the cold-room.

The combined alkaline washings were extracted with petroleum ether, acidified and the liberated fatty acids taken up in petroleum ether. After washing with water, aliquots of this fatty-acid solution were evaporated and tested for activity, and found to give a positive result. The unsaponifiable material was found to be inactive.

The fatty acids thus obtained were often found still to contain traces of Liebermann-positive unsaponifiable material. These proved difficult to remove by extraction methods, but were later readily separated by molecular distillation.

Free fatty acids.

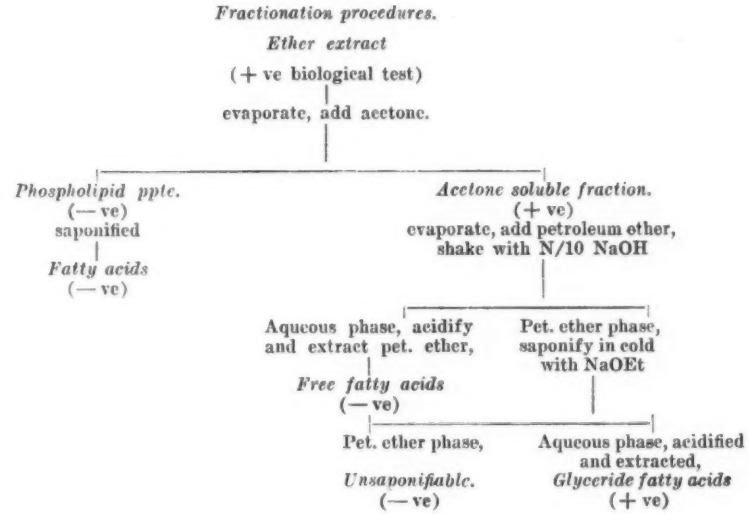
At first it was thought desirable to separate the free fatty acids from glyceride fatty acids. This was achieved by shaking a petroleum ether solution of the acetone-soluble fraction repeatedly with N/10 sodium hydroxide solution. The free fatty acids obtained by acidification of this aqueous phase were tested and found to be inactive. Since this fraction constituted only a small proportion of the total fatty acids, it was not separated in later work.

Phospholipid fatty acids.

Since the phospholipid fraction had been found to be inactive, it was felt that this inactivity might be due to the binding of an active fatty acid in such a manner as to inactivate it. The acetone insoluble fraction was therefore saponified, and the fatty acids extracted, but they proved to be inactive.

TABLE 1.

Fractionation procedures.



Fractionation of fatty acids.

In view of the specific ultra-violet absorption spectra of several of the classes of unsaturated fatty acids (Kass, 1944), a preliminary investigation into the possibility of correlating activity and absorption characteristics was undertaken. The active fatty acid fraction, in optically pure hexane (transparent down to 220 mμ) was examined in the Beckmann quartz photoelectric spectrophotometer. Special precautions were taken to remove traces of interfering solvents.

TABLE 2.

Molecular distillation of crude egg-yolk glyceride fatty acid fraction.

Material	Iodine values*
Crude egg-yolk F.A.	77·5
Residue	88·0
Sublimate	67·0

* Hanus method.

As can be seen from Fig. 1 the active acids (curve 1) differ considerably from the inactive phospholipid acids in the 260—280 mμ, as well as the 230 mμ region.

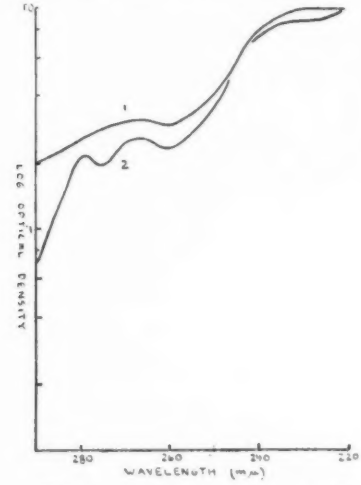


Fig. 1. Curve 1 represents the crude ox spinal cord glyceride fatty acid fraction. Curve 2 the crude phospholipid fatty acid fraction from the same source. The optical density is recorded in terms of an arbitrary maximum density of 1·0. The scale on the graph is logarithmic.

(1) Molecular distillation.

This procedure was used as a preliminary method of purification, although from theoretical considerations complete fractionation was not expected. The fatty acids were volatilised in a chamber connected to a three-stage diffusion pump system, at temperatures from 70–90° C., and condensed on a water-cooled finger. After distillation the sublimate was washed off with petroleum ether.

A typical distillation, run for three days, gave the result shown in Table 2.

(2) Low temperature fractionation.

The fractionation was carried out in boiling tubes (15 × 2·5 cm.) immersed in Dewar flasks (20 × 9 cm.) containing an alcohol-dry ice mixture. Filtration at low temperatures was achieved by the use of a sintered glass filter stick (Jena No. G 2) with a capillary lead-off.

The molecular distillate was dissolved in acetone to form a 30 p.c. solution which was stored in the cold-room at 0° C. The filtrate was adjusted to 10 p.c. concentration and then cooled further, resulting in a series of precipitations.

The results obtained with egg-yolk fractionated after molecular distillation are shown in Table 3.

TABLE 3.

Low temperature fractionation of egg-yolk glyceride fatty acids, submitted to molecular distillation prior to fractionation.

Fraction	Nature	Concn. of F.A. soln.	Temperature	Iodine value	Activity
A	Ppte.	30 p.c.	0° C.	36·0	— ve
B	Ppte.	10 p.c.	— 20° C.	20·5	+ ve
C	Ppte.	—	— 40° C.	47·5	— ve
D	Ppte.	—	— 60° C.	78·0	— ve
E	Soln.	—	— 60° C.	85·0	— ve

Active fraction B constituted about 30 p.c. of the molecular distillate.

Spectroscopic data. In Fig. 2, curve 2 (—20° C.) represents an active fraction, while neighbouring fractions are inactive. Similarity in the 260—280 mμ. band in the three curves is apparent, but the active material has a lower relative density in that region than the other two fractions.

(3) Urea fractionation.

A method of fatty-acid fractionation by means of differential solubilities in urea solutions has recently been developed by Schlenk and Holman (1950). Whilst some initial success has been observed by us in its application, low yields have resulted from emulsion formation, and there have been inconsistencies in the fractions obtained. The results shown in Table 4, obtained from the fractionation of a crude egg-yolk fatty acid preparation, are however worthy of record.

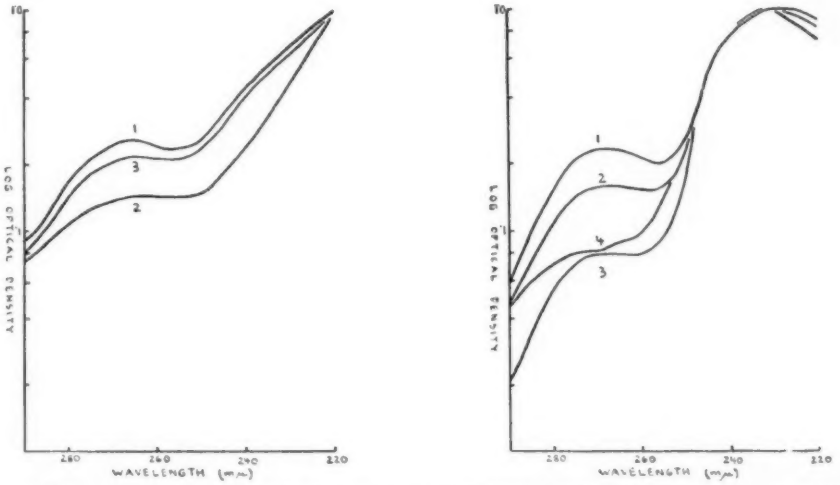


Fig. 2. The curves represent the low temperature fractionated egg-yolk glyceride fatty acid fraction. The crude fraction was submitted to molecular distillation prior to fractionation. Curves 1, 2 and 3 represent fractions precipitated at 0° C., —20° C. and —40° C. respectively. The optical density is recorded in terms of an arbitrary maximum density of 1·0. The scale on the graph is logarithmic.

Fig. 3. The curves represent the urea fractionated egg-yolk glyceride fatty acid fraction. The crude fraction was submitted to molecular distillation prior to fractionation. Curves 1, 2, 3 and 4 represent fractions 1, 2, 3 and 4 mentioned in the text. The optical density is recorded in terms of an arbitrary maximum density of 1·0. The scale on the graph is logarithmic.

TABLE 4.

Urea fractionation of egg-yolk glyceride fatty acids, submitted to molecular distillation prior to fractionation.

Fraction	Nature	F.A./Urea ratio	Iodine value	Activity
1	Ppte.	10 : 3	23·5	—
2	Ppte.	1 : 1	66·0*	+
3	Ppte.	1 : 2		+
4	Soln.	1 : 2	115·0	—
2–I†	Ppte.	1 : 1·3	32·5	+
2–III	Ppte.	1 : 2	40·5	—

* Since fractions 2 and 3 were both found to be active, they were mixed before iodine values were determined.

† Fraction 2 was further subfractionated.

Spectroscopic data. In Fig. 3, curves 2 and 3 represent the active fractions. Although curves 1, 2 and 3 show similarity in the 260—280 mμ region, this factor alone cannot be significant, since fraction 1 is inactive.

TABLE 5.

Extinction data of urea fractions of egg-yolk glyceride fatty acids, submitted to molecular distillation prior to fractionation.

Fraction	$E^{1\ p.c.}_{1\ cm.}$ at 230 mμ	$E^{1\ p.c.}_{1\ cm.}$ at 268 mμ
1	3·76	0·88
2	7·00	0·11
3	7·05	0·06

However, as can be seen from Table 5, active fractions 2 and 3 are much closer to each other in extinction values than they are to inactive 1.

Properties of fatty acid preparations.

It became obvious quite early that special precautions had to be taken to preserve biological activity. All activity was rapidly lost if dry preparations were exposed to air. Susceptibility to oxidation was confirmed by the complete loss of activity following treatment with ozone. Following these observations, preparations were stored as concentrated petroleum ether solutions in the cold-room.

Following the report by Morton, Todd and Laser (1949) of a haemolytic fatty acid in blood and other tissues, active extracts were tested for haemolytic activity, according to Laser's (1949) technique. Haemolysis proceeded rapidly at high dilution.

DISCUSSION.

The precise nature of the neurocletic substance(s) must still be considered uncertain. In view of the loss of activity following even aerial oxidation, it appears certain that the fatty acid is unsaturated; however the iodine values, together with the data from low temperature fractionation suggest that the activity is restricted to acids containing only one double bond. Since oleic acid, with one double bond (see Tables 2 and 3), has an iodine value of 90, the active fractions so far obtained could contain, at most, only 25 p.c. of an active acid possessing one double bond.

From the spectroscopic data it appears that activity is absent from those fractions yielding extinction curves deviating grossly from the flat 268 mμ. maximum. However, it should be pointed out that activity is not always present where this type of curve is encountered. It is obvious that the fractions so far examined have been mixtures, and that no detailed information can be obtained at this stage from spectroscopic studies.

Identity with any of the more common fatty acids, such as those listed by Riemenschneider *et al.* (1938) as being present in egg-yolk has been discounted, since an artificial mixture of palmitic, stearic, oleic and linoleic acids was shown to be inactive. However, acids such as *cis*-vaccenic, recently isolated by Morton

and Todd (1950) and shown to be identical with Laser's haemolytic fatty acid cannot be similarly disregarded, since the neurocletic extracts studied here proved to be actively haemolytic. However, Breusch and Bodur (1950) have discussed the haemolytic activities of a number of fatty acids, and it may be that a wide range of biologically active fatty acids exists.

It is suggested that the release of one or more moderately unsaturated fatty acids, *in the form of glycerides*, from disintegrating nerve-fibres (following Wallerian degeneration) stimulates intact axons and motor end-plates to sprout new processes. Certain tentative suggestions may be made at this stage in regard to the mode of action of the neurocletic substance. Laser (1950) has demonstrated that, apart from its haemolytic activity (i.e. ability to rupture the membrane of the red cell) *cis*-vaccenic acid also has a specific inhibitory effect on bacterial metabolism, which he concludes is due to a selective penetration of the cell membrane, followed by a non-specific inhibition of the succinoxidase system. Booij (1949), in an analysis of the effects of fatty acids and anions on cells, points out that they readily penetrate the plasma membrane, sometimes disorganising it, and in some cases also lower the viscosity of the protoplasm. The presence of a double bond greatly enhances this activity. It seems therefore reasonable to suggest that the specific properties of neurocletin are due to its ability to penetrate the cell membrane—axolemma—and disorganise the axoplasm. This process of penetration would be possible by virtue of the specific structure of the molecule—in this regard it is of interest to note that *trans*-vaccenic acid is far less active biologically than the *cis* isomer.

Lewis (1950) has pointed out that the internal organization of axoplasm is very similar to that of amoeba protoplasm. The axolemma encloses a thin layer of gelled axoplasm (axogel) within which lies the much larger mass of liquid axosol, which is capable of reversible gelation. Advance of the growth cone at the axon tip is conceived as comparable in mechanism to amoeboid movement. It has further been extensively demonstrated (Weiss and Hiscoe, 1948; Weiss, 1945; Young, 1945; Gerard, 1950) that axoplasm, synthesized in the cell body (perikaryon) continually flows distally, and some of these workers suggest that this flow maintains the turgor of the axon. Finally Levi (1926) has demonstrated that micromanipulation of neuroblast cell bodies or axons results in pseudopodial sprouting. This may be attributed to breakdown of the axolemma, due to either traumatic change or initiation of some physiological process of stimulation. It is therefore conceivable that the penetration of the fatty acid or its glyceride into the axon, (by virtue of specific structural properties) locally disturbing the plasmalemmal structure and liquefying the axogel, results in an outflow of axosol, which then reversibly gels at the point of breakdown, thus forming a new pseudopodial sprout. Whether particular points on the cell surface are more sensitive to stimulation, or more penetrable, providing specific points at which sprouting may occur, is open to speculation, but possibly the junction of two Schwann or telolemmal cells may provide such an active point.

Evidence has been presented (van Harreveld, 1947; Koechlin and von Muralt, 1947; Hoffman, 1951; Levi-Montalcini, 1951), indicating that growing or regenerating nerves may be stimulated by chemical substances released some distance away, and in such instances, if the same chemical substance is concerned as has been studied in this work, it is possible that it is released as a water-soluble complex, and therefore capable of extensive diffusion. The similarity of neurocletin to the other chemical substances mentioned above is rendered feasible, since on occasion, the injection of neurocletic extracts into tibialis anterior has resulted in the sprouting of end-plates in gracilis, some distance away. However, intraperitoneal injection of propylene glycol emulsions of extracts, and intravenous injections of Ringer-propylene glycol emulsions of active extracts have consistently failed to evoke sprouting.

SUMMARY.

As a result of attempts to fractionate ether extracts of ox spinal cord and egg-yolk, which had been found to evoke nerve-sprouting, it appears that the active substance is a glyceride fatty acid of moderately unsaturated character—probably possessing one double bond. It is postulated that it acts by disorganising the cell membrane, and liquefying the axogel, resulting in outflow of axoplasm.

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THE HAEMAGGLUTININ OF HAEMOPHILUS PERTUSSIS.

IV. REACTIONS AND ANTIBODY RESPONSE IN BABIES AND CHILDREN FOLLOWING THE ADMINISTRATION OF HAEMAGGLUTININ ADSORBED ON ALUMINIUM PHOSPHATE

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Previous reports have described investigations on the production and properties of pertussis haemagglutinin, its rôle as a protective antigen in mice, the antigenic structure of the haemagglutinating complex, the extraction of haemagglutinin from the bacterial bodies, the adsorption of the extracted principle on aluminium phosphate floccules, and the properties of the adsorbed antigen (Keogh, North and Warburton, 1947; Keogh and North, 1948; Fisher, 1948, 1950; and Warburton and Fisher, 1951). This paper deals with reactions and antibody response in babies following injection of haemagglutinin.

At first, soluble (extracted) haemagglutinin was used (Keogh, Williams, Warburton and Fisher, 1949); later this was changed to the adsorbed antigen, as the latter was found more stable (Warburton and Fisher, 1951). Details of tests in which adsorbed haemagglutinin was used will be described below. The immunizing agent will be referred to as HAPA (haemagglutinin, aluminium phosphate adsorbed).

MATERIALS AND METHODS.

Preparation of HAPA and estimation of serum antihaemagglutinin.

Full details of the methods were given by Warburton and Fisher (1951). Antihaemagglutinin titres are expressed in terms of reciprocal of the highest serum dilution giving a positive result.

Immunization and follow-up.

Groups of babies were immunized in babies' homes in Melbourne and suburbs, and carefully observed for local and systemic reactions. Samples of serum for antihaemagglutinin determinations were obtained by venepuncture. Technical reasons made it necessary to carry out the tests on several small groups of babies rather than on a single large group. In addition to the groups subjected to serological study, a number of babies and children who were not inmates of babies' homes were given injections of HAPA, and observed for after-effects. In every case, inquiry was made into prior infection with whooping cough or immunization against the disease. Children who had had either, were excluded from antibody level studies; however, some who had been immunized previously, were given a boosting dose of HAPA.

The tests covered a period of approximately six months. The same batch of HAPA was used throughout. Mouse tests carried out on this batch before, during and after the clinical trials showed no change in the antigenic potency of the material.

RESULTS.

Local and general reactions in babies following the administration of HAPA.

Thirty-three babies were given an injection of 1 ml. subcutaneously. The local reaction reached its peak approximately 24 hours after injection. It consisted of an area of erythema associated with some subcutaneous thickening. The erythema, the outline of which was at times irregular, faded over the course of the next 24 hours, but induration of the subcutaneous tissues persisted much longer and in some cases the site of injection could be recognized by a small firm nodule a month later—a not uncommon finding with many types of subcutaneous injection.

The average diameter of the erythematous area was 2·5 cm. In three babies no reddening occurred, while the maximum diameter, 6·0 cm., was observed in two babies where the shape of the area of erythema was long and narrow. No attempt was made to measure the size of the zone of subcutaneous thickening, as it was never very marked and its margin was poorly defined. In one child a sterile subcutaneous abscess developed at the site of inoculation and discharged a little thin pus for five weeks; this boy (Group 4, subgroup a, case No. 54) was suffering from congenital heart disease with gross cyanosis, so that poor oxygenation of the tissues may have been a factor in the formation of the abscess.

General reactions in these babies were very mild. The majority showed no symptoms or signs of having received an injection. The rectal temperature, taken three times during the thirty-six hours following the injection, showed no rise in twelve babies; in eighteen it rose to between 99° and 101° F.; in two it exceeded 101°, and in one it reached 102·4° F. Two children vomited and six others were a little drowsy or irritable for the twenty-four hours following injection.

In the five children given 1 ml. HAPA by the intramuscular route, the general effects were similar, but the local reactions were less marked.

Six children who received a standard course of a phase 1 bacterial vaccine had more severe local reactions with local erythema of average size 5·5 cm. × 2·5 cm. with a maximum diameter of 8·0 cm. in two babies. Three of the babies had temperatures over 102° F. rectally, and one baby vomited though no other general reactions were noted.

Reactions to smaller doses of HAPA given subcutaneously, were similar in nature to those following 1 ml. introduced by the same route, but milder. Twelve babies received 0·3 ml. and in six, the dose was repeated. Twenty-one were given 0·1 ml. and of these a second dose of the same size was administered to eleven. Reactions following the second injection which, in four of the babies receiving 0·1 ml., was given 11 weeks after the first one, did not appear more severe than those after the first dose.

There appeared to be no correlation between the severity of the local and general reactions following injection of HAPA.

The effects of 88 injections of HAPA given to seventy-one babies were thus closely observed. The ages of the children varied between 4 and 30 months. As far as could be ascertained, the subjects had no previous experience of *H. pertussis*, either as natural infection or as vaccine.

In addition to the above group, HAPA has been administered to well over 200 children and babies, in doses ranging from 0·3 ml. to 1 ml., using the subcutaneous route. The ages of the subjects varied from 6 months to 14 years; some had received prior immunization against whooping cough. While the previously unimmunized babies reacted to the injection of HAPA in a manner similar to the babies referred to above, in some of the older children and in those

previously immunized against pertussis, the effects were more marked, and consisted of malaise, vomiting, anorexia, as well as local erythema and induration of larger areas. One child, aged nine years, who was given 1 ml. HAPA in a single subcutaneous injection, developed a sterile abscess.

Antihaemagglutinin titrations on sera of unimmunized babies.

54 sera, obtained from babies who had no history of whooping cough or immunization against it, were tested. The babies' ages, at the time of bleeding, ranged from three months to three years.

None of the sera was positive in a titre of 2. Since there was very little whooping cough in Melbourne during the few months which elapsed between the times at which these samples were taken and the beginning of the tests with HAPA, it was not considered necessary to obtain pre-immunization serum samples from the babies in the latter groups.

Antibody response in babies to the administration of HAPA.

Five distinct tests were carried out, each on a different group of babies. The babies within each group were inmates of the same babies' home. Each group was divided into 2 or 3 subgroups of not less, whenever possible, than 5 babies, keeping the age distribution approximately the same in the subgroups.

In a reference subgroup of each group, the babies received 1 ml. HAPA by the subcutaneous route. In Groups 1, 2 and 3, the responses to subcutaneous injections of one and of two doses of 0·1 ml. and 0·3 ml. amounts of HAPA were tested. In Group 4, the effect of 1 ml. HAPA injected by the intramuscular route, was compared with that evoked by the reference dose. In Group 5, the latter was tested against the antihaemagglutinin rise following the injection of a total of 27,000 million organisms of stock phase 1 pertussis bacterial vaccine in three divided doses.

The detailed results of the tests are given in Tables 1 to 5, and the information is summarized in Table 6.

TABLE 1.

Antibody response to subcutaneous administration of HAPA: single doses of 0·1 ml. and 1 ml. (Group 1.)

Sub-groups	Dose	Case No.	Age (Months)	Titre 4 weeks after inj.	Titre 7 weeks after inj.	Titre 11 weeks after inj.	Titre 15 weeks after inj.
a	0·1 ml.	1	6	2	< 2	■	■
a	0·1 ml.	2	6	< 2	< 2	■	■
a	0·1 ml.	3	8	2	< 2	■	■
a	0·1 ml.	4	9	8	4	■	■
a	0·1 ml.	5	10	4	4	■	< 2
a	0·1 ml.	6	12	< 2	< 2	■	< 2
a	0·1 ml.	7	15	4	4	< 2	■
b	1 ml.	8	6	32	16	■	4
b	1 ml.	9	8	8	8	2	■
b	1 ml.	10	9	8	8	2	< 2
b	1 ml.	11	10	16	8	2	< 2
b	1 ml.	12	11	16	16	8	8
b	1 ml.	13	15	32	32	8	■
b	1 ml.	14	16	8	16	4	■

■ = no specimen available.
< = test negative in titre shown. (Highest serum concentration tested.)

TABLE 2.

Antibody response to subcutaneous administration of HAPA: two doses of 0·3 ml., single doses of 1 ml. (Group 2.)

Sub-groups	Dosage	Case No.	Age (months)	Titre 3 weeks after last inj.	Titre 7 weeks after last inj.
a	0·3 ml. once	15	5	< 2	< 2
a	0·3 ml. once	16	4	< 2	■
a	0·3 ml. once	17	8	8	16
a	0·3 ml. once	18	9	8	■
a	0·3 ml. once	19	10	■	< 2
a	0·3 ml once	20	13	< 2	8
b	0·3 ml. twice	21	4	8	16
b	0·3 ml. twice	22	5	16	32
b	0·3 ml. twice	23	8	< 2	16
b	0·3 ml. twice	24	12	16	32
b	0·3 ml. twice	25	10	64	128
c	1 ml. once	26	4	4	16
c	1 ml. once	27	9	8	32
c	1 ml. once	28	11	32	32
c	1 ml. once	29	11	4	16

Where two doses were given the interval between the injections was three weeks. Where a single dose was given its administration coincided with that of the first dose of the babies receiving two injections.

■ = no specimen available.
< = test negative in titre shown. (Highest serum concentration tested.)

TABLE 3a.

Antibody response to subcutaneous administration of HAPA: two doses of 0·1 ml., single doses of 1 ml. (Group 3.)

Sub-groups	Dosage	Case No.	Age (months)	Titre 6 weeks after last injection
a	0·1 ml. once	30	6	< 2
a	0·1 ml. once	31	10	< 2
a	0·1 ml. once	32	6	4
a	0·1 ml. once	33	5	< 2
a	0·1 ml. once	34	4	< 2
b	0·1 ml. twice	35	11	32
b	0·1 ml. twice	36	6	32
b	0·1 ml. twice	37	5	32
b	0·1 ml. twice	38	4	4
b	0·1 ml. twice	39	10	8
b	0·1 ml. twice	40	6	32
b	0·1 ml. twice	41	6	32
c	1 ml. once	42	11	16
c	1 ml. once	43	10	4
c	1 ml. once	44	10	32
c	1 ml. once	45	7	32
c	1 ml. once	46	6	16
c	1 ml. once	47	5	8
c	1 ml. once	48	5	16

Where two doses were given, the interval between the injections was three weeks. Where a single dose was given, its administration coincided with that of the first dose of the babies receiving two injections.

< = test negative in titre shown. (Highest serum concentration tested).

TABLE 3b.

Antibody response to subcutaneous administration of HAPA: two doses of 0·1 ml. injected at prolonged interval. (Group 3.)

Sub-group	Case No.	Titre 6 weeks after 1st injection	Titre 8 weeks after 2nd injection
a	31	< 2	16
a	32	4	16
a	33	< 2	16
a	34	< 2	8

The interval between the two injections was 11 weeks.
< = test negative in titre shown. (Highest serum concentration tested.)

TABLE 4.

Antibody response to subcutaneous and intramuscular administration of HAPA: single doses of 1 ml. (Group 4.)

Sub-groups	Route	Case No.	Age (months)	Titre 6 weeks after injection	Titre 17 weeks after injection
a	Subcut.	49	4	4	8
a	Subcut.	50	5	16	■
a	Subcut.	51	9	32	8
a	Subcut.	52	16	8	4
a	Subcut.	53	20	< 4	2
a	Subcut.	54	30	64	32
b	Intramusc.	55	5	16	16
b	Intramusc.	56	5	8	■
b	Intramusc.	57	7	16	8
b	Intramusc.	58	10	32	16
b	Intramusc.	59	15	8	4

■ = no specimen available.
< = test negative in titre shown. (Highest serum concentration tested.)

TABLE 5.

Antibody response to subcutaneous administration of HAPA, and of stock phase 1 bacterial vaccine. (Group 5.)

Sub-groups	Dosage	Case No.	Age (months)	Titre 6 weeks after last inj.	Titre 10 weeks after last inj.
a	Bacterial vaccine$_1$ 3 doses	60	7	32	16
a	3 doses	61	7	2	< 2
a	3 doses	62	6	4	2
a	3 doses	63	9	2	2
a	3 doses	64	9	2	< 2
b	HAPA, 1 ml.	65	7	32	16
b	HAPA, 1 ml.	66	7	16	16
b	HAPA, 1 ml.	67	7	32	16
b	HAPA, 1 ml.	68	4	4	16
b	HAPA, 1 ml.	69	9	8	16

The babies in one group received 3 doses of stock bacterial vaccine at the following dosage: 5,000, 10,000 and 12,000 million organisms. The interval between the first and second injections was 4 weeks; between the second and the last, 3 weeks. The babies in the other group received 1 ml. HAPA simultaneously with the second injection of those given bacterial vaccine.

< = test negative in titre shown. (Highest serum concentration tested.)

TABLE 6.

Antibody response in babies to the administration of HAPA. (Summarized from Tables 1 to 5.)

Group and subgroup	Agent, route, dose	No. of babies	G.M. of titres (1st test)	Significance	G.M. of titres (2nd test)	Significance
1 (a)	One dose 0·1 ml. HAPA. (S.C.)	7	2·4 (4 weeks)	Difference is highly significant.	1·8 (7 weeks)	Difference is still highly significant
1 (b)	One dose 1·0 ml. HAPA. (S.C.)	7	14·5 (4 weeks)		13·1 (7 weeks)	
2 (a)	One dose 0·3 ml. HAPA. (S.C.)	6	2·3 (3 weeks)	Differences (a) and (b) not quite sig.	3·4 (7 weeks)	Differences (a) and (b) sig. (a) and (c) sig. (b) and (c) not sig.
2 (b)	Two doses 0·3 ml. HAPA. (S.C.)	5	10·6 (3 weeks)		32 (7 weeks)	
2 (c)	One dose 1·0 ml. HAPA. (S.C.)	4	8·0 (3 weeks)	(a) and (c) not quite sig. (b) and (c) not sig.	22·6 (7 weeks)	
3 (a)	One dose 0·1 ml. HAPA. (S.C.)	5	1·3 (6 weeks)	Differences (a) and (b) H. sig. (a) and (c) H. sig. (b) and (c) not sig.		
3 (b)	Two doses 0·1 ml. HAPA. (S.C.)	7	19·5 (6 weeks)			
3 (c)	One dose 1·0 ml. HAPA. (S.C.)	7	14·5 (6 weeks)			
4 (a)	One dose 1·0 ml. HAPA. (intramuscular)	5	11·3 (6 weeks)	Difference not sig.	7·0 (17 weeks)	Difference not sig.
4 (b)	One dose 1·0 ml. HAPA. (S.C.)	6	13·9 (6 weeks)		9·5 (17 weeks)	
5 (a)	Three doses Phase I, bactl. vaccine (S.C.)	5	4·0 (6 weeks)	Difference not quite sig.	2·3 (10 weeks)	Difference highly sig.
5(b)	One dose 1·0 ml. HAPA. (S.C.)	5	13·9 (6 weeks)		16 (10 weeks)	

G.M. = Geometric mean. Significance based on P = ·05 level.

1 ml. HAPA given subcutaneously, caused production of antihaemagglutinin to a demonstrable titre in every case; the highest titre was 64. The geometrical means of the antihaemagglutinin titres in the subgroups receiving this dosage were 14·5, 22·6, 14·5, 13·9 and 13·9 (Groups 1 to 5, Tables 1, 2, 3a, 4 and 5). The mean titres were at the highest levels between 6 and 10 weeks after the immunization; some babies still had measurable amounts of antihaemagglutinin 15 weeks (Group 1, Table 1) and others, 17 weeks (Groups 4, Table 4) after the administration of the dose.

The mean antihaemagglutinin titre after two doses of 0·3 ml of HAPA, given subcutaneously, was 32 at its peak (Group 2, Table 2); after two doses of 0·1 ml. given 3 weeks apart, the mean titre was 19·5 (Group 3, Table 3a). As these results were obtained in different groups, they cannot be directly compared; however, neither figure differed significantly from the mean in the appropriate reference subgroup. The babies receiving single doses of 0·3 ml. and of 0·1 ml. amounts of HAPA, subcutaneously, gave mean titres of 3·4 and of 1·3 (Groups 2 and 3, Tables 2 and 3a). These titres were significantly lower than those fol-

lowing two doses of the corresponding quantities of the antigen, as well as the titres in the reference subgroups.

In four of the babies given one dose of 0·1 ml. HAPA subcutaneously (Group 3, subgroup c, cases No. 31, 32, 33 and 34), the dose was repeated 11 weeks later. After a further 8 weeks, the mean serum antihaemagglutinin titre of these four babies, which had been 1·4 after the first injection, was 13·6 (Table 3b).

Three doses of stock pertussis vaccine, given over 7 weeks resulted, 10 weeks after the last dose, in a mean titre of 2·3, a figure significantly lower than that following the reference dosage, 16 (Group 5, Table 5); the difference between the titres 6 weeks after immunization, 4·0 and 13·9, was not quite significant. There was little difference between the effects of 1 ml. HAPA given by the subcutaneous and the intramuscular routes. The mean titres were 11·3 and 13·9 respectively (Group 4, Table 4).

The ages of the babies receiving 1 ml. HAPA by either route ranged from 4 to 30 months. No correlation could be detected among these subjects between age and antibody response.

DISCUSSION.

The relationship between serum antihaemagglutinin level and protection of mice against intranasally introduced experimental pertussis has been pointed out in previous papers (Keogh and North, 1948; Fisher, 1950); the lowest mouse serum titre at which consistent and firm protection could be demonstrated was about 16 (Fisher and Warburton, 1950; Warburton and Fisher, 1951).

Serum antihaemagglutinin levels of the same order as the protective level in mice were present in babies given 1 ml. HAPA as a single subcutaneous or intramuscular dose, or as little as 0·2 ml. antigen administered in two divided subcutaneous injections spaced from 3 to 11 weeks apart. The effect of repeated doses smaller than 0·1 ml. each, has not been tested. The titres reached their peaks 6 to 10 weeks after the last injection, and, when followed through, antibody was still demonstrable after 15 to 17 weeks. In the one experiment in which a comparison was carried out, HAPA was a more efficient antihaemagglutinin-producing antigen than a stock phase 1 bacterial vaccine; the latter was administered according to a schedule believed to be usual in clinical practice. In this test, the amount of bacterial vaccine given to one baby contained more organisms than were used in the preparation of one dose of HAPA given to the babies in the control group. Since, however, the antigenic potency of bacterial vaccines was found to vary considerably (Fisher and Warburton, 1950), conclusions drawn from this single test cannot be held valid for bacterial vaccines in general.

Toxic reactions, local and general, were, on the whole, slight, and definitely less marked than those usually experienced during the administration of full immunizing courses of bacterial pertussis vaccine. The reactions to HAPA were more marked in the older age groups and in those previously immunized against whooping cough, suggesting the development of allergic sensitivity to a bacterial component present in HAPA. Similarly, reactions to diphtheria toxoid are more severe in adults (Pappenheimer, Edsall, Lawrence and Banton, 1950), presumably because of previous experience of the antigen.

Thus HAPA, given to babies in certain ways, caused comparatively slight side-effects, while raising the serum antihaemagglutinin titre to levels which,

when present in mice, were associated with protection of the animals against lethal doses of *H. pertussis* instilled into the nostrils. As there was no guide available, the mouse protective titre was adopted as the serum level to be aimed at in babies, although it was realized that the results of mouse experiments may not be fully valid when applied to naturally occurring human disease. In protection of human subjects, special conditioning of the antibody producing mechanism may be more important than antibody levels following immediately after the immunization; furthermore, haemagglutinin may not be the only, or perhaps not even the chief, protective antigen. While, therefore, from evidence presented above and in previous papers, it appears likely that HAPA would be an efficient immunizing agent against whooping cough in man, field trials will be required to prove this point.

If, however, HAPA should be proved to be an effective prophylactic against whooping cough in man, it will have marked advantages over current agents used for this purpose, not the least of which being that it is an extremely stable product, of high immunizing potency, which can be accurately standardized *in vitro*.

SUMMARY.

HAPA (pertussis haemagglutinin, aluminium phosphate adsorbed), injected into babies in single subcutaneous or intramuscular doses of 1 ml., or two subcutaneous doses of 0·1 ml. or 0·3 ml. spaced 1 to 2½ months apart, produced mean serum antihaemagglutinin titres between 11·3 and 32. Single subcutaneous doses of 0·1 ml. or 0·3 ml. resulted in significantly lower serum antibody levels.

Local and general toxic effects, observed in over 270 subjects, were mild in babies and young children with no history of previous immunization against whooping cough. The reactions were more marked in older children and in those previously sensitized with *H. pertussis*, but were no more severe than those associated with administration of bacterial pertussis vaccine in customary doses.

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THE ABSORPTION AND METABOLISM OF SUBSTANCES WITH VITAMIN K ACTIVITY UNDER NORMAL CONDITIONS AND AFTER BILIARY OBSTRUCTION

A STUDY IN MAN AND RABBITS, INCLUDING A VITAMIN K SATURATION TEST.

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Although it is established that 2-methyl-1, 4-naphthoquinone or certain derivatives of it are required for the synthesis of prothrombin in the liver, their mode of action is not understood and the metabolism of these accessory food factors has only been partly investigated. As a contribution to the elucidation of these problems, experiments pertaining to the absorption and excretion of certain synthetic vitamin K preparations are reported. Mainly lipoid soluble and water insoluble compounds were used, because it was desired to test the apparent general assumption that lipoid soluble vitamin K preparations require bile for intestinal absorption.

EXPERIMENTAL.

2-Methyl-1, 4-naphthoquinone (menaphthone) was prepared according to Fieser (1940) and 2-methyl-1, 4-naphthohydroquinone-diacetate (acetomenaphthone) was produced from it by reductive acetylation. The latter compound was used in these experiments because of its low solubility in water, and according to Ansbacher *et al.* (1942) its toxicity is one third of that of menaphthone and, finally according to Dam (1942) the compound is approximately as active as Vitamin K_1 when tested in chickens. The drugs were given in gelatine capsules. Rabbits weighing 1·5–2·5 kg. were maintained one week prior to, and throughout the period of observation, on cabbage leaves of which approximately 800 gm. were consumed per day. The animals were kept in metabolism cages which allowed collection of urine with little contamination by faeces. Bacterial influence was minimized by addition of merthiolate to the receiving bottles (final concentration of merthiolate, 1:10,000). In some experiments normal butanol or hydrochloric acid was preferred as preservative.

The common bile ducts of the animals were severed after ligations in two places 6 mm. apart.

Investigations in the post-operative period were carried on until the animals started to lose weight which occurred, in some cases, 40 to 50 days after the operation. After conclusion

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of the experiments the gall bladders were inspected. Results are recorded only from animals which showed complete biliary obstruction. This was verified by X-ray photographs obtained after injection of Neo-hydriol into the distended gall bladder. In the case of humans the drugs were given to volunteers of the age groups from 15–70 years who were either healthy people or patients recovering from diseases not related to vitamin K deficiencies or gastro-intestinal disorders. The patients suffering from obstructive jaundice were inmates of surgical wards of this hospital. Urinary metabolites of the drugs were determined by the Craven reaction for 2-methyl-1-4-naphthoquinone as carried out by Richert (1944).

The following blood components were estimated: Prothrombin (by the technique of Quick), bilirubin, alkaline phosphatase, (King Armstrong units) and fibrinogen. The gall-bladder contents were analysed for bile acids by a modified Pettenkofer reaction and by a fluorometric procedure (Fantl and McQuade, to be published).

TABLE 1.

Urinary excretion of derivatives of menaphthone after oral administration of acetomenaphthone to normal and bile-duct ligated rabbits.

Rabbits' condition	No.	I Aceto-menaph-thone given in mg.	II Excretion product calculated as mg. of menaph-thone X	III Re-covery of drug as per-centage of dose given	Blood constituents			
					Pro-thrombin in p.c.	Bili-rubin in mg. p.c.	Alkaline phos-phatase units	Fibrinogen mg. p.c.
Normal	1	200	52	39	100	0·25	3–5	220–310
Normal	4	200	48	36	100	0·25	3–5	220–310
Normal	6	200	51	38	100	0·25	3–5	220–310
16 days after C.B.D. ligation	4	200	58	44	> 75	2·0	—	—
	6	200	58	44	> 50	1·8	—	—
28 days after C.B.D. ligation	4	200	49	36	100	2·5	—	—
	6 (P)	200	56	42	> 75	1·8	105	—
37 days after C.B.D. ligation	2 (P)	60	12·1	30	> 70	3·5	27	140
	5XX	60	14·6	36	> 80	3·0	26	150

X Prior to the administration of the drug, rabbits maintained on cabbage leaves excreted in 24 hours 0·1 mg. approximately of substances giving a positive Craven reaction.

XX X-ray photograph of the biliary tract and gall bladder of this animal is shown in the attached photograph.

(P) *Rabbit 6*—Pathologist's report on liver is: "Biliary cirrhosis with increased cellularity of portal tracks, some plugs of bile in small bile ducts and early bile duct hyperplasia."

(P) *Rabbit 2*—Pathologist's report on liver is: "The liver shows an increase in portal tract fibrous tissue which contains a few chronic inflammatory cells. There is moderate bile duct hyperplasia and a little fatty degeneration. Otherwise the liver parenchymal cells look 'healthy.' Early biliary cirrhosis."

The results given in the above Table indicate that normal rabbits excreted, in 24 hours after the oral administration of 200 mg. of acetomenaphthone, 30–44 p.c. of compounds giving a Craven reaction (Col. III). D. Richert (1944) found that normal rabbits excreted between 31–42 p.c. of administered menaphthone. A comparison of the normal excretion values with those obtained after ligation of the common bile duct shows clearly that up to six weeks after the operation, intestinal absorption of acetomenaphthone and the urinary excretion of its metabolites are within the normal range. The X-ray photograph of the gall bladder (rabbit 5), taken after the conclusion of the experiment shows the complete blockage of the common bile duct and the great distention of the gall bladder from a normal volume of approximately 1·1 ml. to 18 ml. This condition was observed in all cases although the distention was not always so great. The fluids aspirated from the gall bladders were almost colourless. The bile acid concentration in gall bladder content at the end of the experiment was in rabbit No. 2 less than 0·3 mg. p.c. and in rabbit 5 less than 1·8 mg. p.c. calculated as cholic acid. The normal bile acid concentration in gall bladder bile of rabbits was found to be at least 1,500 mg. p.c.

It is apparent from these figures that only insignificant amounts of bile salts could have reached the intestinal tracts of the experimental rabbits.

With regard to the blood plasma tests, all the figures, excepting prothrombin, are indicative of obstructive jaundice. None of the experimental animals showed a reduction of prothrombin in undiluted plasma. Deviation from the normal prothrombin time could be observed only in specimens diluted to 12·5 p.c. with 0·85 p.c. sodium chloride. It has to be pointed out, however, that the animals developed a reduction in the fibrinogen level. Deutsch *et al.* (1946) and Fantl and Nance (1948) and Fantl (1949) have shown that results of prothrombin tests are influenced by the fibrinogen concentration. Lowering beyond a critical level will delay the coagulation time. Therefore it is very likely that the apparently smaller prothrombin values recorded are minimum figures as indicated in the Table. At any rate the prothrombin reduction was in no case low enough to suggest vitamin K deficiency.

Results obtained after the administration of acetomenaphthone to humans are given in Table 2.

The first eight cases listed in Table 2 include healthy volunteers and patients from medical wards recovering from illnesses other than those affecting the hepatic or gastrointestinal system. With regard to intestinal absorption or metabolism they can be considered as normal. Their urinary excretion in 24 hours ranged from 20–29 p.c. of the 50–60 mg. acetomenaphthone given. It will be noted that the greater part of this quantity was excreted in less than 12 hours. Patients Nos. 9–14 were cases of obstructive jaundice. Prior to operation all of them had, in addition to the clinical symptoms, the biochemical findings associated with this condition. It will be observed that two patients out of three with a very low prothrombin level excreted from 0–7 p.c. of the administered drug when it was given the first time (a). The administration of the drug was repeated a few days later (b) and the urinary excretion was now 14–15 p.c. respectively. The prothrombin level rose slowly to 60 p.c. in the case of Patient No. 10 and to 50 p.c. in the case of No. 11. Patients Nos. 9–14 were operated upon as soon as their prothrombin levels reached at least 60 p.c. of the normal value. Patient No. 13 showed complete blockage of the common bile duct by gallstones whilst the other patients had external obstruction due to carcinoma of the head of the pancreas, or to inflammatory lesions thought to be chronic pancreatitis. In three instances, (Patients Nos. 9, 12 and 13) the contents of the gall bladders were aspirated during operation. Eighty to one hundred ml. of a colourless viscous fluid (white-bile) was obtained from Patient No. 13 which contained less than 3 mg. p.c. of bile acids. The gall bladder of Patient No. 9 contained approximately fifty ml. of a straw coloured fluid with lumpy sediment; the bile acid concentration was less than 30 mg. p.c. Finally, Patient No. 12 had a distended gall bladder estimated to contain

60–80 ml. A dark-green viscous fluid containing 2,000 mg. p.c. bile acids was obtained. These figures indicate an insignificant concentration of bile salts in the first two cases and a normal concentration in the last one. It is perhaps worth mentioning that in no case did the surgeons observe excessive bleeding.

TABLE 2.

Urinary excretion of derivatives of menaphthone after oral administration of acetomenaphthone to humans.

Clinical condition	Aceto-menaph-thone given in mg.	Excretion product calculated as mg. of menaphthone	Recovery of drug as per-centage of dose given	Blood constituents		
				Prothrombin in p.c.*	Bilirubin in mg. p.c.	Alkaline phosphatase units
Normal 1.	50	8·5 (24)	25	80–100	< 0·5	3–15
Normal 2.	58	8·4 (24)	22	80–100	< 0·5	3–15
Normal 3.	54	7 (24)	20	80–100	< 0·5	3–15
Normal 4.	60	11·6 (24)	29	80–100	< 0·5	3–15
Normal 5.	60	11·7 (24)	29	80–100	< 0·5	3–15
Normal 6.	60	8·4 (12) 0 (12)	21	80–100	< 0·5	3–15
Normal 7.	60	5·2 (8) 2·3 (8) 1·0 (8)	21	80–100	< 0·5	3–15
Normal 8.	60	8·3 (8) 1·1 (8) ·4 (8)	25	80–100	< 0·5	3–15
Obstructive jaundice 9.	60	8·1 (12) 3·2 (12)	28	50	24	21
Obstructive jaundice 10a.	60	2·8 (24)	7	20	—	30
Obstructive jaundice 10b	70	6·9 (24)	15	60	—	—
Obstructive jaundice 11a.	60	0 (24)	0	30	42	41
Obstructive jaundice 11b.	60	3·1 (12) 2·6 (12)	14	50	—	—
Obstructive jaundice 12	60	8·2 (12) 1·9 (12)	25	60	7	27
Obstructive jaundice 13a.	60	6·25 (24)	16	30	8	50
Obstructive jaundice 13b.	60	6·4 (24)	16	50	—	—
Obstructive jaundice 14.	60	5·6 (24)	14	25–40	30	55
Liver cirrhosis 15.		9·8 (24)	24	70	—	—
Multiple mye-lomata 16.	60	8·4 (24)	21	30	0·5	6

The figures in brackets refer to hours after administration of drug. If there is more than one bracket the results refer to successive periods.

* The prothrombin estimations were carried out before the administration of the drug.

Fig. 1. Normal rabbit. Neo-Hydriol introduced into the gall bladder through catheter. Neo-Hydriol is seen passing into the duodenum. 0·7 natural size.

Fig. 2. The same as 1. Five minutes after ligation of the common bile duct. Arrow indicates point of ligature. The hepatic branches of the biliary system are outlined and no futher Neo-Hydriol is entering the duodenum. 0·7 natural size.

Patient No. 15 is a case of liver cirrhosis in the terminal stage and yet showed a normal absorption and excretion of acetomenaphthone.

Patient No. 16 is a case of multiple myelomata who was investigated because of haemorrhages. The whole blood coagulation time was delayed and it appeared to be due to a low prothrombin level. It was found, however, that this result was not due to prothrombin deficiency but to the fact that the activity of thrombin was masked by the presence of a powerful thrombin antagonist (Table 3, Col. II). Diluting the plasma with saline to 25 p.c. (Col. I) abolished the effect of the antithrombin and the prothrombin results became normalized.

TABLE 3.

Apparent hypoprothrombinaemia in a case of multiple myelomata.

Whole blood coagulation time Lee White (glass)	I Prothrombin time			II Thrombin inhibition in percentage		
	1	1/2	1/4	1	1/2	1/4
Patient: 12–17 min. Control: 6–10 min.	20″ 14″	20″ 16″	22″ 21″	75–96 p.c. —	50–80 p.c. —	20–50 p.c. —

The figures in Table 3 represent the lower and higher limits of observations made during one month.

The test for antithrombin was carried out in the following manner. To 0·2 ml. of citrated or oxalated plasma kept at 37° C. was added 0·1 ml. of a beef thrombin preparation and the coagulation time determined. Simultaneously with the thrombin clotting time on patient's plasma, normal control plasmas were similarly tested. The thrombin preparation was

adjusted to a clotting time of 16–19 seconds. From a curve relating thrombin concentration and clotting time, the thrombin inhibition was calculated. The figures in the Table are expressed as percentage inhibition.

The patient was under urethane treatment, but the drug was not found to be responsible for the antithrombic property. Although the nature of the thrombin inhibition could not be ascertained heparin could be excluded by the behaviour towards protamin sulphate. In this connection it is important to point out that it is necessary to use very active thromboplastin preparations for the determination of prothrombin because patients suffering from obstructive jaundice may also show inhibition of thrombin activity, i.e. Patients Nos. 9 and 12 inhibited to the extent of approximately 50 p.c. If in such cases brain preparations of low potency are used, erroneously low prothrombin figures are obtained.

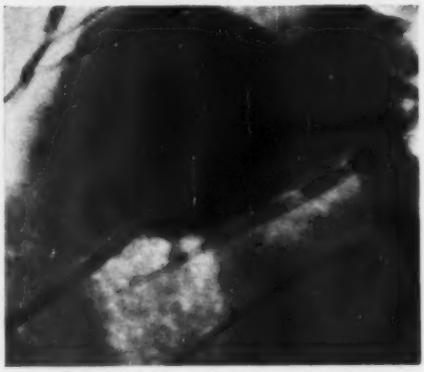

Fig. 3. Rabbit 5 XX. 37 days after ligation of the common bile duct (See Table 1). Grossly dilated gall bladder is outlined. Complete biliary obstruction is seen, no Neo-Hydriol passing into the duodenum. Hepatic biliary system only incompletely filled. 0·7 natural size.

Several of the patients listed showed a smaller urinary excretion of compounds giving the Craven reaction than normal persons. It is, however, doubtful that this subnormal excretion after the first administration of acetomenaphthone could be explained by a deficient absorption. It appears more likely that Patients Nos. 10 and 11 were, on admission, in a state of vitamin K depletion and required larger quantities of the anti-haemorrhagic factor to replenish the tissue stores, hence the low urinary excretion. Some evidence for this assumption can be given by the fact that a second administration of the drug resulted in an excretion not far below the normal range.

The results obtained in the cases of the obstructive jaundice patients suggest the application of 2-methyl-1, 4-naphthoquinone derivatives as an indicator of vitamin K deficiency.

The following technique was adopted: 50–60 mg. of acetomenaphthone are given by mouth and a 24-hour urine specimen or two 12-hour specimens collected over a preservative (Hydrochloric acid, normal butanol or merthiolate). A 25 ml. aliquot is hydrolysed with 2·5 ml. hydrochloric acid ($d = 1{\cdot}17$) and the metabolite determined according to the colorimetric procedure of Richert. An excretion of less than 15 p.c. in 24 hours following the administration of the drug is indicative of vitamin K deficiency. Estimations of the prothrombin level in blood are carried out simultaneously. In the case of deficient excretion the administration will have to be repeated a few days later.

Evidence that the urinary product giving a Craven reaction is 2-methyl-1, 4-naphthoquinone was obtained by the following experiments: The excretion products after administration of the drugs tested could be extracted from urine by normal butanol and could be re-extracted from this solvent by 0·05N sodium hydroxide.

Butanol extracts from urines of three rabbits who obtained a total of 1·2 gm. menaphthone orally were combined. Acid hydrolysis liberated 136 mg. of an ether soluble crystalline product, which, according to the Craven reaction, contained 120 mg. menaphthone. The ether

solution of the product was treated with freshly prepared silver oxide, magnesium sulphate was added and the mixture shaken for 20 minutes. The filtered solution was evaporated to dryness, the residue dissolved in peroxide-free ether and pigments removed by washing with dilute sodium hydroxide. The residue of the ether layer was recrystallized from methanol in the presence of charcoal. Yellow needles with m.p. of 102–103° C. were obtained which did not depress the m.p. of an authentic sample of 2-methyl-1, 4-naphthoquinone.

DISCUSSION.

The necessity of bile for the intestinal absorption of substances with vitamin K activity has been discussed by several workers. In the earlier period of vitamin K research preparations of natural origin were used and the effect on the prothrombin level of blood was the criterion of efficacy. Smith, Warner, Brinkhous and Seegers (1938) found that feeding of alfalfa to bile fistula dogs produced only a moderate rise in prothrombin and the addition of bile salts was necessary for effective treatment. Greaves (1939) produced bile fistulae in rats previously maintained on a vitamin K deficient diet and states that the oral administration of a vitamin K concentrate, together with bile salts led to a rapid recovery of the hypoprothrombinaemia. Butt, Snell and Osterberg (1938) reported that oral administration of an unspecified material containing vitamin K was only effective in raising a low prothrombin level when given together with bile salts. Zuckerman *et al.* (1939) observed a patient with a complete external biliary fistula who was maintained on a diet deficient in vitamin K. The drop in the prothrombin level could be reversed only by the combined oral administration of a vitamin K concentrate and bile. These references, by no means complete, would make it likely that vitamin K was not absorbed unless bile was available. On the other hand, there are observations which are not in agreement with such an assumption. Watkins and Wright (1933) studied a baby with congenital atresia of bile ducts that lived for 15 months, and found the coagulation time of blood normal throughout that period, indicating that the prothrombin level could not have been dangerously low. Finlayson (1937) examined a child with congenital obstruction of the bile ducts that lived for 3½ years. No abnormal bleeding, even during and after exploratory operations, was noted. Quick (1938) found a normal prothrombin level in a two months' old infant with absence of extra hepatic ducts. Sanford and Shmigelski (1942) presented the case of a baby with complete obstruction of the gall bladder and extra-hepatic ducts who lived for six months and whose prothrombin one week before death was 75 p.c. of the normal when tested by the one-stage procedure of Quick and 32 p.c. in the two-stage procedure of Warner, Brinkhous and Smith (1936). Finally, Morse and Schmidt (1941) found that bile fistula rats kept on deficient vitamin K diet absorbed 2-methyl-1, 4-naphthoquinone from the intestinal tract and that deoxycholic acid was not necessary to ensure the absorption of the vitamin.

The results presented here leave no doubt that the intestinal absorption of acetomenaphthone, a water insoluble compound, occurs in the rabbit in the presence or in the complete absence of bile, to the same degree as judged by urinary excretion of its metabolite. In the case of patients suffering from obstructive jaundice distinct differences in the urinary excretion were observed after the first administration of acetomenaphthone. This however was not due to impaired absorption since a second administration of the drug was followed by higher excretion rates. These results indicate that bile is also not required in humans for absorption of acetomenaphthone.

The different opinions regarding the necessity for bile in the intestinal absorption of vitamin K preparations can perhaps be explained by the following considerations: Vitamins K_1 and K_2 have larger molecular weights, 450 and 580 respectively, than acetomenaphthone (258). Efficient absorption in humans and perhaps in dogs may require bile in aiding the transport of the first two substances across the intestinal mucosa but not of the third. Rabbits with a comparatively large intestinal tract are capable of absorption of these compounds without the presence of bile. Further, it should be pointed out that most of the pertinent experimental work has been carried out on pathological material. Hawkins and Whipple (1935) and Hawkins and Brinkhous (1936) observed in dogs with cholecystonephrostomoses spontaneous bleeding 3–5 months after operation and Brinkhous *et al.* (1938) who estimated the prothrombin levels in bile fistula dogs found that it took several months for the prothrombin level to drop after elimination of bile. This observation has been confirmed by Quick and Collentine (1951). It is known from the work of Gutman *et al.* (1940) and Drill *et al.* (1945) that these operations produce secondary changes in liver as indicated by bromsulphalein retention and elevation of serum alkaline phosphatase. Finally, the question of vitamin K storage has to be considered. According to Dam (1942) no appreciable storage of vitamin K occurs in the chicken. Of interest in this connection are observations of Flynn and Warner (1940). These workers ligated bile ducts of rats who were fed a vitamin K deficient diet. Although the hypoprothrombinaemia of the experimental animals could be alleviated by parenteral administration of menaphthone, prothrombin fell within 3 days to 20 p.c. of the normal level when no more vitamin K was given. Further, Smith *et al.* (1938) report that the rapid rise in prothrombin following bile salts and vitamin K (alfalfa) ceased in all cases as soon as the dietary supplement was discontinued. Evidently vitamin K was used promptly and little or none was stored. Why did it then take several months for the prothrombin level to drop after exclusion of bile? Probably because the animals developed liver damage. It is possible that an impaired liver requires larger amounts of vitamin K than a normal one.

The suggested saturation test should make it possible to obtain information regarding the patient's store of vitamin K. However, this test does not indicate the ability of the liver to produce prothrombin. When the urinary excretion of menaphthone is combined with the estimation of the prothrombin level in blood, this function of the liver can also be determined. It will be recalled that the assessment of the last-mentioned activity has been carried out by F. Koller (1941), by Hansen *et al.* (1943) and by P. N. Unger *et al.* (1948).

SUMMARY.

The oral administration of the diacetate of 2-methyl-1, 4-naphthohydroquinone (acetomenaphthone) to normal humans and rabbits is followed by the urinary excretion of a derivative of the drug yielding in the case of rabbits 30–40 p.c. and in case of humans 20–30 p.c. of 2-methyl-1, 4-naphthoquinone.

Complete obstruction of the biliary flow produced by ligation and severance of the common bile duct in the rabbit does not influence intestinal absorption and urinary excretion of acetomenaphthone, and no significant decrease in the prothrombin level can be detected. In patients suffering from obstructive jaundice, marked variations from nil to normal excretion following the first administration of acetomenaphthone, have been observed. In the case of low urinary excretion, a second administration of the drug gave higher excretion rates. From these results, it is concluded that bile is not required for the intestinal absorption of acetomenaphthone in humans. A vitamin saturation test for the detection of vitamin K deficiency depending on the oral administration of acetomenaphthone and estimation of the urinary excretion, has been developed.

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THE INFLUENCE OF SODIUM SALICYLATE AND ACETYL SALICYLIC ACID ON THE RELEASE OF HISTAMINE IN ANAPHYLAXIS

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It is well known that salicylic compounds are of value in the treatment of recognized allergic conditions such as rheumatic fever and asthma. It has also been shown that salicylate will prevent precipitate formation when added to a mixture of egg albumin and antibody *in vitro* (Coburn and Kapp, 1943). Jager and Nickerson (1947) report sodium salicylate prevents antibody formation to H and O antigens in rheumatic fever patients injected with typhoid vaccines. Perry (1941) was not able to affect the development of agglutinins following T.A.B. vaccination in the presence of acetyl salicylic acid administration. In an attempt to investigate this problem further we have investigated the effect of salicylates on the release of histamine and slow-reacting substance (Kellaway and Trethewie, 1940) in anaphylaxis.

Methods.

Lung perfusion.

Guinea-pigs were sensitized by the subcutaneous injection of 15 mg. crystalline egg-albumin (kindly prepared by Dr. Trautner). Injections were made in sterile saline (30 mg. albumin per ml.). The animals were stunned by a light blow on the head 6–9 weeks after the sensitizing injection and the lungs perfused as described by Kellaway and Trethewie (1940). In certain instances sodium salicylate or acetyl salicylic acid (100, 50, 25, 12·5 or 6·3 mg. per 100 ml.) was dissolved in the Tyrode used for perfusion of the lung. In these experiments antigen was dissolved in a similar solution immediately prior to injection. When the blood was washed out of the lungs the rate of perfusion was set at 1 ml. per minute, and after collection of the first sample (3 minutes) egg-albumin dissolved in Tyrode was injected from a syringe into the pulmonary artery in a volume of 0·5 ml. after the flow was first interrupted. The flow was re-established after 1½ minutes. Perfusion samples were then collected for 10 minutes (S_2) and for another 5 minutes (S_3). The volumes were measured and all the samples boiled immediately and quickly cooled.

Histamine assay.

Histamine was assayed as histamine acid-phosphate. The samples were assayed for histamine and S.R.S. on the isolated jejunum of the guinea-pig (Trethewie and Kellaway, 1940). In some instances selective inhibition of the response of the gut to perfusion samples was

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carried out by injecting Benadryl into the bath before assay in such a dose that the response to acetylcholine was still brisk while that to moderate doses of histamine was abolished.

The influence of salicylic compounds on the response of the gut to histamine was also tested by adding 1 or 2 mg. sodium salicylate, salicylic acid or acetyl salicylic acid to the bath after the gut was responding maximally to histamine. After obtaining a constant response to added histamine, salicylate was injected into the bath and left in contact 3–4 minutes while the succeeding dose of histamine was injected into the bath.

EXPERIMENTAL.

(a) The effect of salicylate on the histamine response of the gut.

When 1 mg. salicylic acid was added to the bath (vol. 5 ml.) the response to 0·1 μg. histamine was reduced thereafter to about 60 p.c. and the gut responses to further histamine inoculations (without further addition of salicylic acid) were further depressed and this was still evident after 35 minutes. This phenomenon was obtained regularly.

On the other hand when 1 mg. or 2 mg. acetyl salicylic acid were injected into the bath while any rhythm of the gut, if present, was usually abolished, the response of the gut to histamine was little affected though sometimes small after-changes in the excitability of the organ are noted. These have been described previously for venom by Feldberg, Kellaway and Holden (1938).

Similarly 1–2 mg. sodium salicylate produced little effect on the excitability of the gut. Sometimes a very slight reduction in the response to histamine was noted. The amount of salicylate added to the bath in perfusion samples (e) was 0·05–1 mg.

(b) The output of histamine and SRS from the sensitized guinea-pig lung following the injection of crystalline egg-albumin (controls).

It has been shown previously that histamine is liberated from the isolated Tyrode perfused lung of the guinea-pig following the injection of egg-albumin when the animal has been sensitized previously (Bartosch, Feldberg and Nagel, 1932). Further Kellaway and Trethewie (1940) showed that in addition to histamine, "SRS" (a slow-reacting smooth muscle stimulating substance) was also liberated. In the present experiments a control experiment was at first always carried out immediately preceding each "salicylate" experiment so that for adequate comparison the antigen injected was obtained from the same solution. Also this ensured that the duration of the "sensitization time" was the same in each pair of animals. It soon became evident that the difference in output of histamine was so striking between these two groups (control and salicylate perfused) that this was not necessary. In one instance the control perfusion followed immediately on the salicylate perfusion to ensure that "subclinical" desensitization from contamination was not operative. In this instance histamine was still liberated from the control lung.

In all but one of these control perfusions, histamine was liberated into the perfusate following the injection of albumin. In the other experiment there was a reasonable output in the drainage fluid only. This may have been due to insufficient ventilation of the lung required to squeeze out the tissue fluid into the vessels. It was not likely to be due to non-sensitization of the guinea-pig since in a number of instances a portion of gut was removed from the guinea-pig and tested for sensitivity to albumin by suspending it in a bath of Tyrode and exhibiting the antigen. In every instance when this was carried out the gut was found to be sensitive. Control gut did not respond. Since the experiments were carried out in pairs at random, one with and one without salicylate, errors due to difference in sensitivity are randomized between the two groups and statistical analysis can show the probability of chance accounting for any difference in histamine output.

The findings in one experiment are illustrated in Fig. 1 (lower panel). At B is shown the response to 1 ml. of perfusate S_2 obtained 0–10 minutes after the injection of 5 mg. egg-albumin in control experiment (no salicylic acid added to perfusion fluid) No. 10, and at C to 0·8 ml. of S_3 obtained 10–15 minutes after the injection. The responses in each case were immediate and equal to 0·04 μg. and 0·01 μg. histamine respectively. Thus the samples contained the equivalent activity of 1 in 5 mill. and 1 in 16 mill. histamine. Sample A (Fig. 1) (prior to injection of antigen) was without effect on the gut and thus contained no estimable histamine.

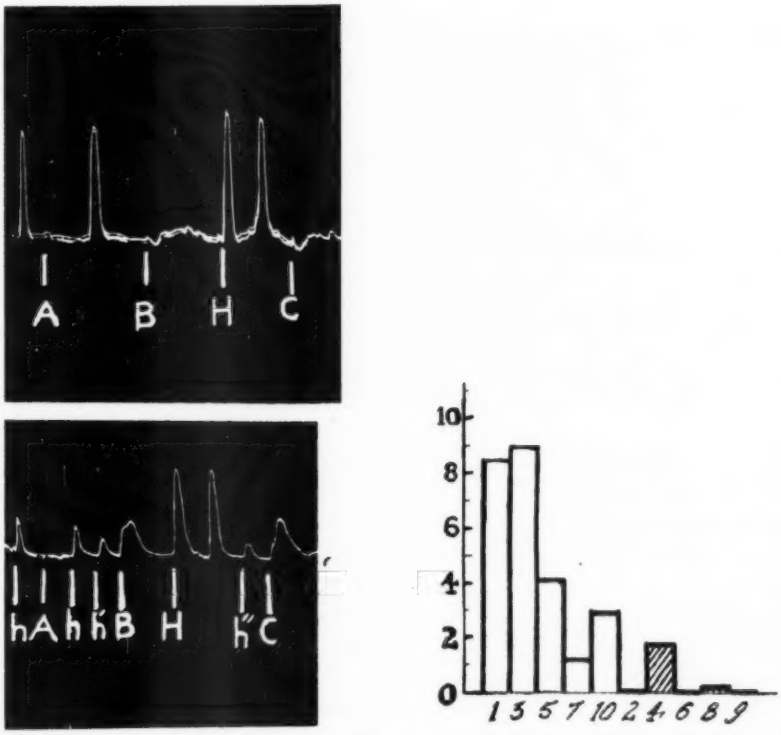

Fig. 1. Responses of the isolated jejunum of a guinea-pig to perfusate from guinea-pig No. 9 (upper panel) and No. 10 (lower panel). Upper panel: at A 0·5 cc. S_1 added to the bath, at B 0·5 cc. S_3 added to the bath, at C 0·5 cc. S_2 added to the bath. Lower panel: At H 0·1 μg., at h 0·05 μg., at h' 0·02 μg., and at h" 0·01 μg. histamine added to the bath. At A 0·6 ml. S_1 added to the bath, at B response to 1 ml. S_2 and C response to 0·8 ml. S_3. Samples left in contact 10". The unlettered contractions are to 0·1 μg. histamine. Time in minutes. Details in text.

Fig. 2. Comparison of output of histamine with and without added sodium salicylate (100 mg./100 ml.) in the perfusion fluid. Ordinate: Output expressed as 1,000 x μg. histamine liberated per mg. albumin injected per μg. histamine content of lung per g. Abscissae; Animal No. 1, 3, 5, 7, 10: control pigs; 2, 4, 0, 8, 9 (cross-hatched); salicylate perfused pigs.

TABLE 1.

Comparison of histamine output with salicylate and control perfusions.

Pig No.	Salicylate perfusion				
	Histamine content (μg./gm.) lung	Dose of albumin (mg.) injected	Output of histamine (μg.) in sample		
			S_2	S_3	d
2	25	1·5	—	—	—
4	67	5	0·60	—	—
6	40	2·5	tr	—	
8	25	5	—	—	↑0·02
9	42	5	—	—	—

tr = trace.

TABLE 1 (Continued).

Comparison of histamine output with salicylate and control perfusions.

Pig No.	Control perfusion				
	Histamine content (μg./gm.) lung	Dose of albumin (mg.) injected	Output of histamine (μg.) in sample		
			S_2	S_3	d
1	12·5	1·5	tr	tr	0·16
3	53	5	1·73	0·09	0·57
5	70	2·5	0·60	0·116	
7	30	5	—	—	0·18
10	20	5	0·24	0·24	0·02

tr = trace.

The output of histamine in all the control perfusions is recorded in Table 1 (No. 1, 3, 5, 7 and 10). It is noted that the total output of histamine varied from 0·16–2·38 μg. with an average output of 0·75 μg. for the 15 minute perfusion. (In the case of salicylate perfusions it varied from 0·00–0·06 μg., with an average output of 0·012 μg.) The amount of antigen injected (which varied from 1·5 to 5 mg.) was kept the same for each pair of lungs perfused and so for comparison we have divided the output by the dose injected. Further, since it has been shown in another form of tissue injury that the output of histamine varies with the histamine content of the organ (Trethewie, 1939) and since there is a considerable variation in the histamine content of the lungs, this figure has also been divided by the histamine content (μg./gm.) of the lung and multiplied by 1,000. When this is done we obtain the figures in Table 2. This procedure reduces the figures to more comparable values. The output of histamine in the control perfusions so expressed varies from 1·2–9·0 with an average of 5·2.

Though convincing evidence has already been presented (Bartosch, Feldberg and Nagel, 1932) that the stimulating substance which produces immediate contraction of the gut, liberated in this type of experiment is histamine, we have produced further evidence with the

antihistamines now available. Using a gut which was not sensitive to S.R.S. and which was treated with Benadryl, it was found in the instances tested that the immediately stimulating substance liberated was inhibited in producing a response from the gut in a similar degree to histamine. This treated gut still produced a brisk response to acetylcholine.

TABLE 2.

Output of histamine expressed as μg. × 1,000 per mg. albumin injected per μg. histamine content per gm. lung.

Pig No.	Salicylate perfusion	Pig. No.	Control perfusion
2	0·0	1	8·5
4	1·8	3	9·0
6	0·0	5	4·1
8	0·2	7	1·2
9	0·0	10	3·0

(c) Output of histamine when the lung is perfused with sodium salicylate in Tyrode.

When the lung was perfused with salicylate in a concentration of 100 mg./100 ml. Tyrode the output of histamine was largely inhibited. In Fig. 1 (upper panel) at A is shown the response to 0·5 ml. of the control sample S_1 from pig No. 9. At B and C is shown the response to 0·5 ml. of S_2 and S_3 respectively, obtained after the injection of antigen which, if anything, shows a temporary initial relaxation and then a very slight S.R.S. response appended. Thus histamine, which produces immediate contraction, was not detected in these samples. In the one instance where there was a reasonable output in the perfusate (No. 4) the antigen was injected after being dissolved only in Tyrode which would dilute greatly the salicylate present in the tiny lung. It is not surprising therefore, that histamine was liberated in the first perfusion sample, though notably it was absent from later samples. In one other experiment, histamine, in small amount only, was liberated in the drainage fluid. In both of these samples assay of the histamine was difficult because of the presence of slow-reacting substance which had a short latency.

It appeared that the inhibition of output of S.R.S. was not quite so striking as that to histamine, e.g. in experiment 9, (Fig. 1, upper panel) some S.R.S. is noticed to be liberated while histamine was absent. It is worth noting here that S.R.S. is liberated by ether more readily than is histamine (Kellaway and Trethewie, 1939). The output of histamine as assessed by gut assay was as recorded in Table 1. It is noted that the output of histamine is negligible.

When the output of histamine is reduced to the unit as in Table 2, the figures yield an average output of 0·2 μg. largely contributed to by the "salicylate-free" injection of albumin. These figures are compared with the controls in Fig. 2. A striking difference in output is noted between the groups (open columns, controls; cross-hatched, salicylate perfusions).

(d) Output of histamine when the lung is perfused with Tyrode containing acetyl salicylic acid.

The solubility of acetyl salicylic acid in Tyrode is such that it was difficult to dissolve 100 mg. per 100 ml. This was effected only by warming the mixture to 36° C. When the lung, weighing 2 gm. of a sensitized guinea-pig weighing 310 gm., was so perfused no histamine was liberated following the injection of 5 mg. albumin. This lung contained 39 μg. histamine/gm. after perfusion. In another experiment a lung containing 110 μg. histamine per gm. was perfused with Tyrode containing 50 mg. acetyl salicylic acid p.c. Following the injec-

tion of 5 mg. albumin, S.R.S. was liberated in the perfusate but no histamine. The drainage fluid, however, contained 1:16 mill. histamine in 1·5 ml. When 25 mg. acetyl salicylic acid were dissolved per 100 ml. in the Tyrode used for perfusion a doubtful trace of histamine (possibly 1:120 million) was liberated in the third sample following the injection of 5 mg. albumin, but owing to the presence of S.R.S. both before and after the injection of albumin this could not be confirmed as histamine. The drainage fluid contained similarly a doubtful trace of 1:120 million histamine. The lung contained 30 μg. histamine/gm. after perfusion. When 13 mg. acetyl salicylic acid/100 ml. Tyrode was perfused there was a considerable output of histamine following the injection of albumin. There was 1 in 9·2 million in 9·1 ml. in the first sample after injection and 1:5 million in 1·9 ml. in the 2nd sample after injection. The drainage fluid contained 1:1·5 million histamine and the lung contained 65 μg. histamine/gm. Thus a concentration of 13 mg. acetyl salicylic acid per 100 ml. produced negligible inhibition of the release of histamine in anaphylaxis. Higher concentrations are effective.

(c) Output of histamine when the lung is perfused with Tyrode containing sodium salicylate in reduced concentration.

When 50 mg. sodium salicylate per 100 ml. was used no histamine was liberated following the injection of 5 mg. albumin though there was an output of S.R.S. When 25 mg. sodium salicylate p.c. was used in a lung containing 77 μg. histamine per gm. no histamine was detected in the first sample collected following the injection of 5 mg. albumin while in the next sample there was a considerable output of S.R.S. which made it difficult to decide whether histamine was also liberated. If the immediately contracting substance present were histamine an output of 1:8 million histamine was present. Similarly with the drainage fluid it apparently contained 1:18 million histamine but the presence of S.R.S. made this very difficult to determine. When 12·5 mg. sodium salicylate/100 ml. in Tyrode was perfused through a lung containing 21 μg. histamine/gm. histamine was only detected in the drainage fluid and in a concentration of 1:8 million though the volume of this sample was so small that the estimation was difficult. When 6·3 mg. sodium salicylate per 100 ml. Tyrode were perfused in a lung containing 67 μg. histamine/gm. 1:25 million histamine was detected in the first sample following the injection of 5 mg. albumin and the combined 2nd sample and drainage fluids contained 1:6 million histamine.

DISCUSSION.

It has been shown that salicylate neutralizes diphtheria and tetanus toxins without destroying the antitoxogenic property (Birkhaug, 1931). However, since a saturated solution was used, Smith suggests protein denaturation may have occurred (Smith, 1947). We have shown in the experiments reported in this paper that salicylate compounds (sodium salicylate and acetyl salicylic acid) inhibit the release of histamine from the perfused sensitized lung of the guinea-pig which release normally occurs following the administration of the appropriate antigen.

The above findings may be significant with regard to salicylate therapy in rheumatic fever. Rheumatic fever is considered to be an allergic reaction to streptococcal infection. It is undoubted that the pyrexial effect in rheumatic fever is neutralized by adequate salicylate therapy. Quantitative analysis yields further information. We have been able to show that histamine is practically completely inhibited in its release from the sensitized guinea-pig lung when the antigen is injected provided the perfusing fluid contains 100 or 50 mg.

sodium salicylate per 100 ml. When the concentration falls to 25 mg. per 100 ml. histamine begins to appear though some degree of inhibition is still noticed even with 12·5 mg. per 100 ml. When the concentration falls to 6·3 mg. per cent. no inhibition is detected. S.R.S. (slow reacting substance) is also inhibited in its release but slightly less readily than histamine. For favourable therapeutic response in rheumatic fever it is found that 35 mg. sodium salicylate per 100 ml. is required (Peters, 1949). The close agreement in the figures for histamine inhibition in anaphylaxis in the guinea-pig and favourable clinical response in rheumatic fever in humans may not be simply fortuitous. (We find that with somewhere between 25 and 30 μg. salicylate/100 ml. histamine release is completely inhibited.) The conclusion is likely that either histamine or some other substance released in anaphylaxis is responsible for the pyrexia.

Since phagocytosis is not a well developed function of the Aschoff cell which ultimately gives rise to the crippling cardiac fibrosis of rheumatic fever, and since the only known function of histamine in cellular reaction (other than vasodilatation and permeability effects) is to stimulate reticulo-endothelial cells to phagocytic activity, it could be concluded that in the absence of any other effect of salicylate such therapy would have no effect on the fibrosis. Such would seem to be the case. Many paediatricians hold to the opinion that salicylate therapy has no effect on this fibrosis.

When acetyl salicylic acid is used in the perfusing fluid inhibition of the output of histamine in anaphylaxis is complete with 100 mg. and 50 mg. per 100 ml. and practically complete with 25 mg. acetyl salicylic acid per 100 ml. With 12·5 mg. per 100 ml. there was a considerable output of histamine. Now it has been found by Perry (1941) that 2–3 gm. acetyl salicylic acid per day were without appreciable effect on the development of agglutinins following T.A.B. vaccine innoculation. In view of our findings and the immunity results referred to above it may be that the failure to protect was due to an insufficient concentration of acetyl salicylic acid. Were the whole amount of daily drug to be absorbed into the blood stream immediately and kept there for 24 hours it would give a concentration of 50 mg. per 100 ml., but this makes no allowance for the saturation of the tissues which was always carried out in our experiments. Uniform distribution by the body of such a dose would give a concentration of 4 mg. per 100 gm. for immediate absorption. The concentration in the whole of the body water would be similarly 7 mg./100 ml. This concentration as we have shown is insufficient to inhibit histamine release.

However, the inhibition of antibody production is a different matter from inhibition of antigen antibody reaction. The findings of some workers suggest that both the production of antibodies and the antigen-antibody reaction are inhibited. We have shown that the release of histamine is inhibited. It may be that salicylate compounds block the antibody receptor surface of the cells

which would account for a proportionate effect in relation to concentration as we have shown though admittedly the concentration for complete inhibition is high. This would, nevertheless, also account for suppression of production of antibody by the cell surface in sensitization if the precursor structure of the cell lipo-protein which would form antibody is blocked by salicylate. In this way equally the antigen antibody reaction would be inhibited as the linkages to take part in the reaction would be blocked.

SUMMARY.

Sodium salicylate and acetyl salicylic acid when perfused through the sensitized guinea-pig lung inhibit the release of histamine and S.R.S. following the injection of the sensitizing antigen.

The significance of these findings is discussed, particularly in relation to the effective concentration of salicylate.

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THE EFFECTS OF LYMPHATIC OBSTRUCTION AND OF POSTURE ON THE ABSORPTION OF PROTEIN FROM THE PERITONEAL CAVITY

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The results of experiments in which plasma and diluted plasma were introduced into the peritoneal cavity of various animals showed that the absorption of protein into the blood-stream was rapid and almost entirely by way of lymphatics (Courtice and Steinbeck, 1950a and b; 1951). Moreover, nearly all the protein appeared to be absorbed through the peritoneum covering the diaphragm into the underlying lymph capillaries, whence the lymph passed through the mediastinum largely by the parasternal lymph vessels to the apical lymph nodes adjacent to the thymus. From these nodes efferents passed usually to the right lymph duct. It was also shown in the anaesthetized cat that increased respiratory movement accelerated protein absorption.

During the absorption of plasma from the peritoneal cavity of unanaesthetized rabbits and guinea-pigs, some lymph leaked from the parasternal lymph vessels into the mediastinum and pleural cavities as though the apical lymph nodes were acting as a bottle-neck to the increased flow of lymph which was being moved on by the rythmical movements of the respiratory muscles, especially the diaphragm. It was therefore decided to ascertain how the absorption of protein would be affected by ligation of the parasternal lymph channels.

The effect of posture on absorption from the peritoneal cavity has been controversial since Fowler (1900) first recommended the inclined pelvis-down position for the treatment of peritonitis. The fact that the lymphatic absorption of protein is mainly through the diaphragmatic peritoneum suggested that the effect of posture be further investigated in animals.

METHODS.

Rabbits, rats and guinea-pigs were used. To ligate the parasternal lymph channels, small transverse skin incisions were made on either side at the level of the middle third of the sternum in rabbits under initial nembutal anaesthesia, and in guinea-pigs and rats under ether. A curved ligature-carrying needle was then passed through the intercostal muscles and pleura behind the sternum from one side to the other, the needle entering and leaving the thoracic cavity just lateral to the costo-chondral junction. The ligature was then firmly tied,

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so obstructing the two large parasternal lymph vessels which run with the internal mammary artery and vein.

After ligature of these lymphatics 20 ml./kg. of fresh, homologous heparinized plasma, to which 4 mg./kg. of the blue dye T1824 had been added to label the protein, was introduced into the peritoneal cavity by the same procedure as in earlier experiments. The animals were then allowed to recover from the anaesthetic and were later killed at various time intervals, rats and guinea-pigs with coal-gas and rabbits with an overdose of intravenous nembutal, and the amount of protein and of fluid absorbed determined.

In the experiments on the effects of posture, rabbits were used. Under initial nembutal anaesthesia fresh, homologous, heparinized plasma, 2, 6 or 20 ml./kg. body weight to which 4 mg./kg. body weight of the dye T1824 was added, was introduced into the peritoneal cavity as before. Animals were then allowed to recover and each animal was kept in a rabbit-box, in one group horizontal and in the other pelvis-down at an angle of 45° to the horizontal.

All analytical procedures used were the same as described in earlier experiments.

RESULTS.

The effect of ligation of the parasternal lymphatics.

In rabbits the rate of protein absorption was assessed by determination of the dye concentration in the circulating plasma 1, 3 and 5 hours after the intraperitoneal injection of the dyed plasma, and by the measurement of the remaining fluid and protein in the peritoneal and pleural cavities at the termination of the experiment. Average results of these experiments are given in Table 1.

TABLE 1.

The absorption of homologous plasma from the peritoneal cavity of rabbits with intact lymphatics and with parasternal lymph vessels ligatured. 20 ml./kg. plasma plus 4 mg./kg. T1824 introduced into the peritoneal cavity. Animals killed at 5 hours.

	Plasma injected		Fluid remaining				Dye in circulating plasma mg./l.		
			Peritoneal cavity		Pleural cavity				
	ml./kg.	Protein gm. p.c.	ml./kg.	Protein gm. p.c.	ml./kg.	Protein gm. p.c.	1 hr.	3 hr.	5 hr.
Lymphatics intact									
Mean (5)	20	6·85	3·2	4·25	0·3	3·67	6·0	31·9	36·7
S.E. mean			± 1·5		± 0·02		± 0·1	± 8·4	± 2·3
Lymphatics ligatured									
Mean (6)	20	5·94	8·4	4·70	4·0	4·56	4·9	9·5	11·5
S.E. mean			± 1·5		± 0·11		± 1·1	± 2·2	± 3·0

The rise of the dye concentration in the circulating plasma shows that with the parasternal lymphatics ligated the rate of absorption of dye-protein into the blood stream is much less than in control animals. Although these figures do not give an exact estimation of the amount of protein absorbed because of the subsequent leakage of dye-protein from the blood capillaries, it would seem that in the control group the rate of absorption is about 3 times that in the group with ligated parasternal lymphatics. Figures obtained from the determination of the fluid remaining in the peritoneal and pleural cavities agree with these rates of absorption.

Of the 20 ml./kg. plasma injected, the average net absorption in 5 hours was 16·5 ml./kg. in the controls and 7·6 ml./kg. in the other group. From the concentrations of protein in the injected plasma and in the remaining peritoneal and pleural fluid, only 11 p.c. of the injected protein was unabsorbed in the control group in 5 hours, whereas in the group with ligated parasternal lymphatics 62 p.c. of the injected protein remained in the peritoneal and pleural fluid at this time. These differences observed in the two groups are statistically significant.

The average results of similar experiments in rats are given in Table 2. Groups of rats, with lymphatics intact and with the parasternal lymphatics ligated, were killed at 3, 5 and 8 hours after the intraperitoneal injection of dye-plasma. The rate of protein absorbed into the blood was evidently much less when these lymphatics were ligated than in control animals, the results being statistically significant. Experiments on a small group of guinea-pigs gave similar results to those observed in rabbits and rats.

TABLE 2.

The absorption of homologous plasma from the peritoneal cavity of rats with intact lymphatics and with parasternal lymph vessels ligatured. 20 ml./kg. plasma plus 4 mg./kg. T1824 introduced into the peritoneal cavity of each rat.

Hours		Plasma injected		Fluid remaining				Final dye concentration in circulating plasma mg./l.
				Peritoneal cavity		Pleural cavity		
		ml./kg.	Protein gm. p.c.	ml./kg.	Protein gm. p.c.	ml./kg.	Protein gm. p.c.	
3	Lymphatics intact							
	Mean (18)	20	6·66	1·5	5·25	0·2	3·37	40·8
	S.E. mean			± 0·06		± 0·01		—
	Lymphatics ligatured							
	Mean (12)	20	6·66	10·8	5·55	3·4	4·47	18·5
	S.E. mean			± 0·22		± 0·10		—
5	Lymphatics intact							
	Mean (9)	20	5·98	0·4	—	0·0	—	48·0
	S.E. mean			± 0·10		—		—
	Lymphatics ligatured							
	Mean (2)	20	7·17	8·3	5·23	4·1	4·28	33·0
8	Lymphatics intact							
	Mean	20	—	0·0	—	0·0	—	—
	Lymphatics ligatured							
	Mean (2)	20	7·17	0·6	4·56	7·5	5·29	35·0

In all these experiments with ligated lymph vessels the post-mortem appearances were striking. The parasternal lymph channels were deep blue as far as the ligature and blue fluid was seen spreading diffusely from these vessels below the ligature with a resultant gross oedema of the anterior mediastinum and free fluid in the pleural cavities. An excess of blue pericardial fluid was also sometimes observed. It was evident that these lymph channels normally played a major rôle in the removal of protein from the peritoneal cavity, and that if they were blocked the paravertebral lymph vessels and the pleural lymphatics removed protein either directly from the diaphragm or after it had first passed from the parasternal vessels into the anterior mediastinum and pleural cavities.

These experiments did not show that no protein was absorbed directly into the blood stream. To prove that the protein was all, or practically all, absorbed by lymphatics in the rat as had previously been shown in the cat, the right lymph duct and the thoracic duct were ligatured in a small group of rats anaesthetized with nembutal, while in the controls the operative procedure was the same save that the lymph ducts were not actually tied. The volume of dyed rat plasma introduced into the peritoneal cavity was the same as before. In 3 hours the dye levels in the circulating plasma were 19·5 and 24·0 mg./litre in two controls, and 0·9, 0·0 and 0·0 mg./litre in three animals with the lymphatics ligatured. Although some of the dye-protein had left the peritoneal cavity in the latter group, it had diffused out into the anterior mediastinum and pleural cavities and practically none had been absorbed into the circulation. It seemed, therefore, that in the rat as in the cat the protein was all or nearly all absorbed into the lymphatics.

TABLE 3.

The dye concentration in the circulating plasma of rabbits after the intraperitoneal injection of 2, 6 and 20 ml./kg. of homologous plasma containing 4 mg./kg. of the dye T1824. Results are given for groups of animals kept horizontally or inclined at an angle of 45°, pelvis down.

Plasma injected ml./kg.		Dye concentration in circulating plasma mg./l.		
		1 hr.	3 hr.	5 hr.
2	Horizontal			
	Mean (6)	13·6	32·5	35·3
	S.E. mean	± 2·3	± 2·6	± 3·0
	Inclined			
	Mean (5)	2·1	18·1	25·9
	S.E. mean	± 0·6	± 2·6	± 2·8
6	Horizontal			
	Mean (7)	13·9	26·2	28·9
	S.E. mean	—	± 4·2	± 3·8
	Inclined			
	Mean (7)	3·6	11·5	15·2
	S.E. mean	—	± 5·2	± 6·4
20	Horizontal			
	Mean (5)	13·7	26·7	31·2
	S.E. mean	—	± 4·4	± 4·2
	Inclined			
	Mean (4)	3·1	8·9	17·3
	S.E. mean	—	± 2·7	± 2·7

The mean figures at 1 hour with the 6 and 20 ml./kg. doses were obtained from pooled aliquots.

The effect of posture.

Different volumes of plasma, 2, 6 and 20 ml./kg., were introduced intraperitoneally in order to see whether posture would affect the absorption of protein when a small amount of fluid was present and mainly confined to the pelvis in the inclined position and when the volume of fluid was much greater and more than filled the pelvis. The best measure of the protein absorption in these experiments is the level of dye in the circulating plasma. In Table 3 these dye levels are compared in the two groups for each of the three volumes of plasma used. It is clear that in all cases the rate of absorption of dye-protein was slower

in the inclined position than in the normal, horizontal posture. This was especially so during the first hour after the introduction. The absorption of fluid also agreed fairly closely with these findings. In 5 hours with the introduction of 6 ml./kg. 85 p.c. had been absorbed in the horizontal group and 35 p.c. in the inclined group, while with 20 ml./kg. 74 p.c. had been absorbed in the horizontal group and 42 p.c. in the inclined. At 5 hours all the fluid was absorbed in both groups after the introduction of 2 ml./kg.

From the post mortem appearance, it seemed that most of the absorption took place through the diaphragm even though the fluid was injected with the animal in the inclined position and this position maintained. In the time of the experiment there was little or no blue staining of the lymphatics and nodes of the peritoneum except that covering the diaphragm and the vessels draining this region. Even in the inclined position the fluid reached the diaphragm and was then absorbed into the lymphatic channels passing through the thorax. The degree of staining of the diaphragm, however, was different in the two groups, being obviously less in the inclined animals. In addition, with 20 ml./kg. an average of 1·0 ml./kg. of dyed fluid was found in the pleural cavities in the horizontal group, as has been described in previous experiments, whereas in the inclined animals no pleural fluid was found. These observations, therefore, suggest that in the inclined position, although most of the protein was absorbed through the diaphragm the rate of absorption was somewhat slower than in the normal posture.

DISCUSSION.

Evidence suggests that once protein molecules escape from the circulation into the tissue fluid, they can be returned to the blood only by way of the lymphatics whereas small molecules can diffuse freely either way through the blood capillary membrane. Drinker, Field and Homans (1934) demonstrated in the dog that if the lymph vessels of the hind leg were blocked over a long time by the repeated local injections of quinine hydrochloride and crystalline silica, elephantiasis of that leg developed. This does not prove that no extravascular protein at all can get back through the blood capillary, but it is evident that the inward passage must be even more difficult than the outward passage of protein through the capillary membrane. Courtice and Simmonds (1949) and Courtice and Steinbeck (1950a) by cannulating the thoracic and right lymph ducts in cats showed that nearly all the protein introduced into the pleural or peritoneal cavities could be collected in the lymph. Always, however, a little protein entered the blood stream by some other route. It is probable that this small amount passed by other lymphatic communications with the venous system. The other possibility is that some of the protein molecules passed directly into blood capillaries. Since the first possibility cannot be definitely eliminated it is difficult to prove that no protein at all can re-enter the blood capillary.

The present experiments confirm this conception that at least the great majority of protein in the peritoneal cavity is absorbed by the lymphatics. In those experiments with the parasternal lymph vessels ligated, the fluid entered the lymphatics and the rhythmically contracting diaphragm forced the lymph out of the main lymph channels leading eventually to massive oedema of the anterior mediastinum and free fluid in the pleural cavity. If normally some

protein could diffuse back through the blood capillary membrane, it might be expected that more protein would have passed into the blood capillaries of the diaphragm in these circumstances of increased pressure in the lymphatics. When the thoracic and right lymph ducts were tied in three rats, a small amount of protein reached the blood stream in one animal, but no measurable quantity was observed in the other two. A considerable amount of lymph, however, leaked out into the mediastinal tissues because of the back pressure in the lymphatics.

It seems evident, therefore, that there is a one-way passage of protein through the blood capillary membrane, the return to the blood stream being by way of the lymphatics. Since naturally-occurring ascitic fluid in man from whatever pathological cause contains protein in varying concentrations (James, 1949), it would appear that the lymphatic pathways from the diaphragm must play an important part in the removal of this fluid. The experiments of Cunningham (1927), who studied the effect of ligation of the parasternal lymph vessels in rats on the absorption of Prussian blue in 5 p.c. dextrose from the peritoneal cavity, do not give an accurate picture of the removal of naturally occurring fluids. Cunningham interpreted his results as showing that such an obstruction had no effect on the absorption of isotonic solutions from the peritoneal cavity and that absorption of such solutions took place into the blood-vascular system. Prussian blue, however, has a small molecule and will be absorbed differently from the large protein molecules of ascitic fluid.

In the present experiments the post-mortem picture of diffusion from the ligated lymphatics in the thoracic cavity resembles that normally seen after intraperitoneal injection of 20 ml./kg. of plasma, only in an exaggerated form. This effect with completely blocked lymphatics supports the theory that normally after the introduction of fluid into the peritoneal cavity the apical lymph nodes may temporarily be partially blocked with cells and so lead to the leakage of lymph into the mediastinal tissues (Courtice and Steinbeck, 1950b; 1951). This leakage is probably aided by the negative pressure in the thorax. The dye concentration in the free fluid is always much greater than that in the circulating plasma and slightly less than that in the injected fluid in the peritoneal cavity, so it is evident that this fluid comes from the lymphatics. In the early stages the lymph leaks out along the line of the parasternal lymph channels and then diffuses behind the whole of the sternum. It is unlikely that lymph would diffuse through the relatively thick walls of the parasternal trunks, but the back pressure may render the valves of tributaries incompetent, so allowing the lymph to pass back along these tributaries and out through the local lymph capillaries. Thus, whereas there is only a one-way permeability of the blood capillary membrane for proteins, protein molecules can pass either way through the membrane of the lymphatic capillary.

The ease with which protein is absorbed through the diaphragmatic peritoneum compared with the peritoneum elsewhere is remarkable. Fowler (1900) made use of this fact in his treatment of peritonitis in human patients by posture.

He maintained that in the pelvis the lymphatics were fewer than in the diaphragm and soon became blocked with thrombi, so that if the fluid could be kept in the pelvis absorption would be minimized. Dandy and Rowntree (1914), however, injected isotonic salt solution containing phenolsulphonephthalein into the peritoneal cavity and maintained that active absorption occurred from the peritoneal cavity in all postures. The absorption in the pelvis-down position was 15 p.c. less than in normal or head-down positions but no adequate explanation was given. The absorption of phenolsulphonephthalein which is a small molecule will not necessarily be by way of the lymphatics and so will give no indication of the pathways or rate of absorption of a protein-rich fluid such as is found in peritonitis or in most types of ascites.

The present experiments show that in rabbits the lymphatic absorption of protein is slower in the pelvis-down posture. This is so because the fluid less easily reaches the diaphragm, for it seems that whatever the posture, protein is mainly absorbed through the diaphragmatic peritoneum. The interesting observations of the inclined animals are not so much that protein absorption is slower than in animals in the normal posture, but that the fluid in the pelvis does not remain there for long. Some forces, maybe due to the movement of the diaphragm and intestines, propel the pelvic fluid upwards to the diaphragm where it is absorbed into the underlying lymphatics.

SUMMARY.

The effect of ligation of the parasternal lymph vessels on the absorption of protein from the peritoneal cavity has been investigated in rabbits, rats and guinea-pigs.

The results show that protein is absorbed into the diaphragmatic and then the mediastinal lymphatics, but because of the lymphatic blockage the protein leaks out again into the mediastinum and pleural cavities. From here absorption takes place into the pleural lymphatics.

With ligation of the thoracic and right lymph ducts in the rat so blocking the main lymphatic entries into the blood stream, practically no protein reaches the circulation but considerable mediastinal oedema and free pleural fluid are observed.

These experiments support the conception of a one-way permeability of the blood capillary membrane to protein but a two-way permeability of the membrane of the lymph capillary.

The absorption of plasma protein from the peritoneal cavity was slower in rabbits kept inclined pelvis-down at 45° to the horizontal than in animals kept horizontal. In both groups the dye-protein appeared to be absorbed mainly through the diaphragmatic peritoneum.

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FURTHER OBSERVATIONS ON THE DETERMINATION OF MOLYBDENUM IN PLANT AND ANIMAL TISSUE

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An examination of the thiocyanate method for the determination of molybdenum enabled us to establish the optimal conditions for its application to biological material (Dick and Bingley, 1947).

The method consisted essentially of destruction of organic matter by wet digestion and, after dilution and the addition of the necessary reagents, the extraction into amyl alcohol from the aqueous phase of an iron-thiocyanate complex of the molybdenum. Determination of the colour intensity of this solution gave a measure of the amount of molybdenum present.

In the course of several years work, during which large numbers of molybdenum determinations have been made, certain modifications have been introduced to overcome difficulties encountered with some types of samples. Further, it has been found possible to effect some simplification of the procedure which facilitates the handling of large numbers of samples without loss of accuracy or specificity.

With the modifications introduced the method can be applied to the determination of molybdenum in the blood of normal sheep which contains something of the order of 0·5 to 1·0 μg. of molybdenum per 100 ml. of blood.

In view of the increasing interest in the rôle of molybdenum in plant and animal physiology and in the determination of molybdenum in biological material, it is thought desirable to present here the modified method.

EXPERIMENTAL.

Factors affecting specificity.

Interference by titanium. It was noted in our original paper that the presence of titanium introduced an interfering yellow colour which had an appreciable absorption at 465 mμ. This interference could be controlled to some extent by washing the amyl alcohol extract of the molybdenum complex with a dilute aqueous solution of stannous chloride.

The conditions for the formation of this yellow complex have been investigated further. As it could be expected that the formation of titanium complexes would be repressed in the

presence of fluoride, the system containing varying amounts of molybdenum and titanium, and in which the coloured molybdenum complex was developed in the presence of varying amounts of sodium fluoride, was examined in greater detail.

When amounts of titanium ranging from 500 to 2,000 μg. were present in the system, it was found that simple "washing" of the amyl alcohol solution of the molybdenum complex was not completely effective in removing the interfering colour due to titanium. However, if sodium fluoride had been added to the aqueous phase before colour development, a single washing of the amyl alcohol resulted in complete removal of the titanium complex.

It was found that 2 ml. of a 5 p.c. solution of sodium fluoride was necessary to prevent the interference when 2,000 μg. of titanium were present. The addition of up to five times this amount of fluoride was without effect on the colour of the iron-molybdenum thiocyanate complex.

Interference by iron. When attempts were made to apply the method to the determination of molybdenum in blood it was found that all the iron present could not be held in the reduced state by concentrations of stannous chloride within the limits previously determined for stability of the molybdenum thiocyanate complex during the first extraction. However, it was found that the concentration of the stannous chloride in the wash solution could be raised to a level sufficient to reduce all the iron from 25 ml. of blood without adversely affecting the coloured complex. Moreover, when the concentration of stannous chloride in this wash solution was raised from 0·2 p.c. to 0·8 p.c., it was found that the generally accepted practice of saturating the solvent wtih stannous chloride and thiocyanate before extraction became not only unnecessary but undesirable because the films of stannic chloride at the interface made the separation of the phases difficult.

Factors affecting accuracy of determination.

Primary standard. In the preparation of transmission curves with solutions containing known amounts of molybdenum, the use of ammonium molybdate as a primary standard has been found to be unsatisfactory because of the variable composition of different batches of this salt.

It is now our practice to use pure dry molybdenum metal dissolved in sulphuric acid with a trace of nitric acid, diluted with water, slight excess of ammonia added and then made up to volume with water.

Addition of molybdenum. With samples containing very small amounts of molybdenum, more accurate results can be obtained by the addition of known amounts of molybdenum (as standard solution) in order to bring the optical density of the coloured complex in amyl alcohol into the range of most accurate reading on the spectrophotometer.

Standard temperatures. Amyl alcohol expands by approximately 1 p.c. for each 10° C. rise in temperature. This would introduce a serious error in the determination of small amounts of molybdenum especially when resorting to the practice of adding comparatively large amounts of molybdenum as indicated in the last paragraph.

Another effect of changes of temperature is the altered solubility of amyl alcohol in water. This decreases by approximately 0·4 p.c. for the 10° C. rise in temperature between 15° and 25° C. It will be noted that this would introduce an error in the same direction as the expansion of the alcohol with rising temperature.

It has, therefore, been considered necessary to equilibrate the alcohol and aqueous phases at constant temperature (25° C.) before removing the alcohol layer, and to adjust the temperature of the alcohol solution of the molybdenum complex (to, say, 30° C.) before reading the optical density.

Details of Modified Method.

Preparation of sample.

Animal tissues (organs, faeces, wool etc.) and plant material. An appropriate amount of dry, ground tissue is weighed into an 8 in. × 1 in. "Pyrex" tube and the organic matter destroyed by digestion with sulphuric, nitric and perchloric acids as described by Eden and Green (1940).

Blood. When using the 8 in. × 1 in. "Pyrex" tubes in vertical racks, it has been found most satisfactory, with large volumes of blood (up to 25 ml.), to add the blood dropwise, using a "Pyrex" all-glass syringe with a glass needle" attached, to the tube containing the boiling acids. By the time the addition of the sample is completed the total volume is considerably reduced and the digestion can be continued in the ordinary way without any fear of the frothing which is normally so troublesome in digesting blood.

Development of colour.

After digestion is completed and vigorous boiling has been carried out to remove the last traces of perchloric acid, the sample is cooled, diluted with 5 ml. of distilled water and again boiled to destroy nitroso-sulphuric compounds. If the sample contains less than 5 μg. of molybdenum, 5 ml. of the standard molybdenum solution, containing 1 μg. Mo per ml., is accurately added by pipette. Two drops of methyl orange indicator and then sufficient ammonia (S.G. 0·880) to neutralise the residual sulphuric acid are added. The sample is then made just acid with 6N hydrochloric acid and 5·25 ml. of the acid are added in excess to give a final concentration of 3 p.c. HCl. Then 2·0 ml. of 5 p.c. aqueous solution of sodium fluoride and 1 ml. of the standard iron solution (0·7022 gm. $FeSO_4.(NH_4)_2SO_4.6H_2O$ per litre) are added. The volume is now adjusted to 32 ml. with water, and 4 ml. of 20 p.c. potassium thiocyanate solution are added. The system is then mixed thoroughly and 1·5 ml. of 20 p.c. stannous chloride in 4 p.c. hydrochloric acid added. After a further mixing an accurately measured 10 ml. of iso-amyl alcohol (B.P. 130–132° C.) is added. The tube is then stoppered and shaken vigorously for 30 seconds. It is allowed to stand in a water or air bath at 25° C. for 20 minutes with occasional shaking during temperature equilibration. The aqueous phase is drawn off with a hair pipette attached through a reservoir to a water pump.

Then 25 ml. of a solution of stannous chloride (4 ml. of 20 p.c. diluted to 100 ml.) are added and shaken gently for 15 seconds. The tubes are returned to the constant temperature bath and gently agitated occasionally during temperature equilibration. When reduction of all ferric thiocyanate is complete, the alcohol layer is transferred to a centrifuge tube and centifuged to remove water.

The tube is held in a water bath at 30° C. prior to reading the transmission at 465 mμ.

Blanks and standard solutions, as added to the sample, are run concurrently and the amounts of molybdenum are read off from a calibration curve similarly prepared with a series of standard solutions.

SUMMARY.

Further work on the determination of molybdenum in biological material has made it possible to extend the range of materials on which determinations may be made and to increase the accuracy of the determination by eliminating certain sources of error. These errors are discussed and full details of the modified method are given.

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A PLEURODYNIA VIRUS PATHOGENIC FOR INFANT MICE[1]

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The Coxsackie group of viruses first described by Dalldorf *et al.* (1949) and further investigated by Melnick *et al.* (1949) has been mainly studied in the United States, where numerous isolations from a variety of conditions, including cases described as non-paralytic poliomyelitis, pleurodynia, or influenza, have been made (Huebner *et al.*, 1950; Howitt, 1950a, b; Melnick *et al.*, 1950). The widespread occurrence of these viruses in America was further demonstrated by the detection of neutralizing and complement-fixing antibodies in sera collected from individuals in widely separated areas (Melnick, 1950). Coxsackie viruses have also been found in Canada (Rhodes *et al.*, 1950). Few reports of these viruses have come from other parts of the world, although sera from Denmark and Alaska contained Coxsackie antibodies (Melnick, 1950), while Hopkins (1950) and Findlay and Howard (1950) in England referred to the association of Bornholm disease with the Coxsackie virus. In Australia, Stanley and Hayes (1950) recorded the isolation of viruses pathogenic for infant mice from cases of non-paralytic poliomyelitis in Sydney, New South Wales.

As part of a general programme of investigation of poliomyelitis and related conditions in South Australia, we are investigating various materials for the presence of the Coxsackie viruses. Although numerous specimens have been examined these viruses have not yet been detected here in non-paralytic poliomyelitis, but a virus pathogenic for infant mice has been isolated from a case of pleurodynia which occurred during a small epidemic late in 1950. Atkinson (1951) briefly recorded this isolation. The general characteristics of this virus, which resembles the viruses of Dalldorf's group B, and the group III viruses of Pappenheimer *et al.* (1950) are described in this paper.

EXPERIMENTAL.

Isolation of the virus.

The virus was isolated from throat washings of the patient (D.W.H.) taken in physiological saline on the third and fifth days after development of symptoms of pleurodynia. The washings were treated with penicillin (1,000 units/ml.) and streptomycin (5 mg./ml.) and 0·01–0·02 ml. inoculated intracerebrally and 0·02–0·03 ml. intraperitoneally into day old mice. Three weeks old mice were also inoculated intracerebrally with 0·03 ml. Weakness and dragging of the limbs were apparent in the infant mice from the sixth to ninth day, and most of the affected animals died. No effect was detected in the three weeks old mice.

[1] This work was carried out under the aegis of the South Australian Advisory Committee on Poliomyelitis, with the aid of a grant from the Government of South Australia.

The first isolation was designated PL4 and the second isolation PL5. Muscle or brain suspension of PL4 or PL5 from affected animals was inoculated as before into day old mice, and produced stunting of growth and weakness in three days with death in three to six days. After six to eight passages both PL4 and PL5 killed day old mice within three days. Muscle and brain suspensions of PL4 and PL5 were filtered through Seitz EK pads and the bacteriologically sterile filtrates produced effects in infant mice directly comparable with those due to the unfiltered material.

Characteristics of the virus.

Cold resistance. The virus remained viable in throat washings and in infant mouse muscle stored for six to nine months in an electric refrigerator at —22° C. When tested in day old mice the stored throat washings (PL4 and PL5) and infant mouse muscle (PL4) produced results comparable to those obtained with the fresh material.

Resistance to antibiotics. Penicillin (1,000 units/ml.) and streptomycin (5 mg./ml.) were regularly used in early specimens from which the virus was isolated. Both positive throat washings (PL4 and PL5) were treated in this way. Infant mouse muscle (PL4 and PL5) also regularly infected day old mice after treatment with these antibiotics.

Effects of ether on muscle suspensions. Infant mouse muscle suspensions of PL4 were allowed to stand with ether overnight in the refrigerator. After removal of the ether the suspensions produced effects in infant mice comparable to those obtained with the untreated preparation.

Pathogenicity for mice. Both PL4 and PL5 regularly infected day old mice and enhancement of virulence followed serial passage up to six passages. In subsequent passages both brain and muscle suspensions of PL4 and PL5 regularly killed day old mice in about sixty hours. The same preparations inoculated in doses of 0·02 ml. intracerebrally failed to produce any symptoms in three weeks old mice. In younger mice the time of onset of symptoms and of death depended upon age. Two day old mice were more resistant than day old mice; symptoms often failed to appear until the third day after inoculation, and death generally occurred from the sixth to the ninth day. Six- and eight-day old mice were still more resistant. Many showed symptoms four to six days after inoculation, and some animals died in nine to fourteen days, but some survived.

By intraperitoneal inoculation a limited pathogenicity was demonstrated in weaned mice. About four days after injection with 0·2 ml. of 10 p.c. infant mouse muscle suspension of PL4 at least half of the animals appeared ill with ruffled fur, hunched backs and weakness, and several died. Further deaths occurred in this group during the following month. Similar results were obtained with PL5. The hunching was the most striking symptom in these sick animals.

Reduction of the amount of virus delayed the onset of symptoms in day old mice, which developed tremors and weakness of the limbs or flaccid paralysis in three to four days, and showed marked stunting as compared with normal litter mates. They died from the fifth to the ninth day. Stunting of growth, in fact, was a marked feature of virus action, and was particularly noticeable in survivors of mice inoculated when six to eight days old. At five to six weeks these survivors appeared reasonably healthy but were extremely small when compared with uninoculated litter mates. In one or two day old mice surviving inoculation for any period longer than four days, stunting and poor development were also obvious, and the infected animals were easily distinguished from the controls (Fig. 1).

Both PL4 and PL5 readily infected infant mice by either the intraperitoneal or the intracerebral route. The LD_{50} of muscle preparations of the eighth passage of PL4 by the intracerebral route in day old mice was approximately 10^{-6}. Difco nutrient broth was used throughout as the suspending fluid.

No cross infection was detected in cages containing infected and normal infant mice of the same litter.

Histology of the mouse lesions.

Infant mouse material.

For histological study the animals were inoculated intracerebrally when one day old, and were fixed in Susa, after opening the body cavities and removing the skin, cranium and limbs. The limbs and cranium were fixed separately. The lesions found were of considerable variety, and tended to differ from animal to animal, according partly to the LD_{50} of the material, and also to the length of time which elapsed between the inoculation and the death of the animal.

Fig. 1. Moribund stunted infected mouse between two litter-mate controls (11th day).

Fig. 2. Early focal necrotic lesion of muscle fibre.

Muscles. Inflammatory lesions of muscles were found quite frequently, resembling in their general features those previously described (Dalldorf *et al.*, 1949; Melnick and Godman, 1951). The muscles most prominently affected were those of the shoulder girdle, next most frequently those of the hind limbs. Muscular lesions were often found in animals which showed no obvious paralysis, but merely stunting and general weakness. The earliest lesion was a focal coagulation necrosis of a short length of muscle fibre, which became slightly swollen and granular and stained more intensely with eosin (Fig. 2). This was accompanied by a proliferation of sarcolemmal nuclei, and later by oedema and a sparse mononuclear infiltration between the fibres of the affected muscle (Fig. 3). The earliest lesion was not evident until the third or usually the fourth day after inoculation. Animals dying or sacrificed in a shorter

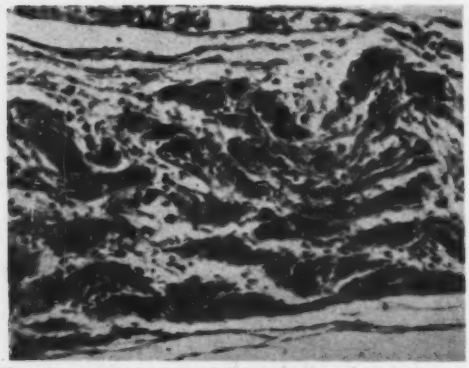

Fig. 3. Sparse mononuclear infiltration between fibres of affected muscle.

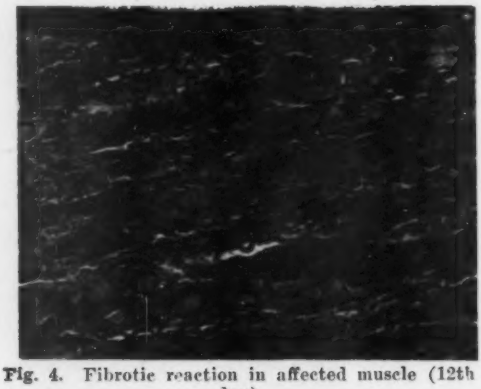

Fig. 4. Fibrotic reaction in affected muscle (12th day).

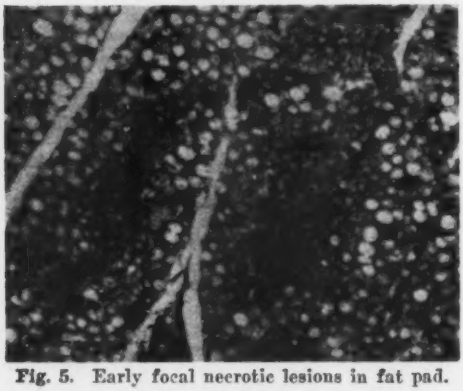

Fig. 5. Early focal necrotic lesions in fat pad.

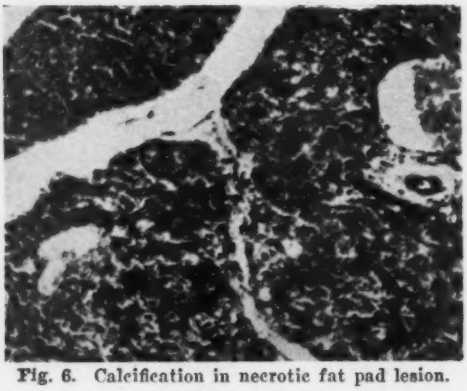

Fig. 6. Calcification in necrotic fat pad lesion.

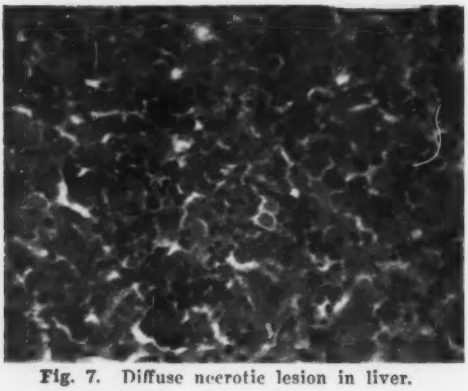

Fig. 7. Diffuse necrotic lesion in liver.

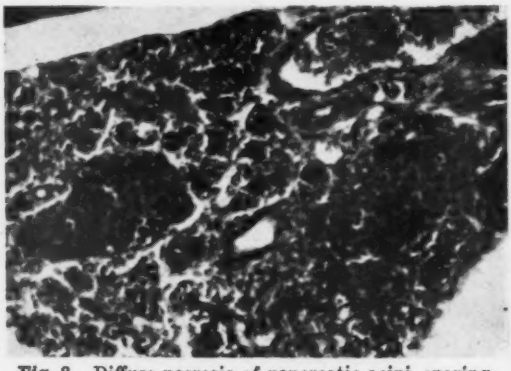

Fig. 8. Diffuse necrosis of pancreatic acini, sparing islets and ducts.

time showed no obvious muscular lesions. By about the twelfth day after inoculation, the inflammatory reaction had given way to a fibrous tissue response (Fig. 4).

Diffuse myositis was seen on one occasion in a single animal of the third passage in a control series inoculated with normal mouse muscle. Further passage however, failed to produce similar lesions. This occurrence, resembling those described by Howitt (1950b), is being further investigated.

Lungs. No special pathological changes, apart from occasional mild interstitial thickening, were seen in the lungs. Emphysema was usual, small areas of collapse were not uncommon, and there was generally an intense vascular engorgement. Similar changes were however, seen in uninoculated controls which were also killed by asphyxiation.

Heart. No lesions were seen in the heart of any of the animals.

Fat pads. Focal necrotic and inflammatory lesions in the fat pads were seen in several animals. The earliest lesion was visible in one animal fifty hours after inoculation, and in other animals lesions were very evident by the fourth and fifth day. The fat was affected in numerous scattered foci. The first change was a granular degeneration of the cytoplasm of a group of adipose tissue cells, with some crowding together (Fig. 5). Very quickly an acute inflammatory reaction developed and by the fifth day calcification was usually evident, commencing as fine scattered flecks which later coalesced (Fig. 6).

Liver. In animals dying on the third to fifth day after inoculation with material from the eighth passage a widespread loss of alignment of the liver cords was evident, with an eosinophilic degenerative change in the liver cells. Pink staining macrophages were prominent in the sinusoids in these cases (Fig. 7). Diffuse focal haemopoietic activity was a feature in the livers of all animals except those which survived until about the tenth day or longer.

Pancreas. Combined with diffuse hepatic necrosis there was in addition in several cases a diffuse or focal pancreatic necrosis, affecting the acini of the organ, but sparing the islets and ducts (Fig. 8). This was accompanied by a sparse inflammatory reaction.

Thymus. In two animals inoculated with crude pharyngeal washings from the same case an intense diffuse pyknotic degeneration of thymocytes occurred, but this change was not seen in any other animals.

Brain. No definite inflammatory lesions were seen in either the brain or spinal cord, although in two animals there was haemorrhage, possibly traumatic, over the posterior aspect of the spinal cord. Most animals, however, died or were killed before the ninth to twelfth day, at which time, with other neurotropic strains, cerebral changes have usually been most evident (Pappenheimer *et al.*, 1950).

Weaned mouse material.

In all adult mice examined the pancreas showed oedema with intense chronic inflammatory infiltration, and disappearance of most of the acini. Islets, too, were scanty, but some were preserved, as were ducts. In one animal there was a moderate infiltration of chronic inflammatory cells in the portal tracts of the liver, though the parenchymatous cells of the organ showed no obvious changes. No definite myositic lesions, or changes in other organs, were seen.

DISCUSSION.

As no type Coxsackie viruses are available in Australia, we have not been able to fit this virus into any of the published American classifications. Detailed histological studies of the lesions produced by viruses pathogenic for suckling mice have been made by Pappenheimer *et al.* (1950) and by Melnick and Godman (1951). The virus at present under discussion seems to resemble those of the third group of Pappenheimer *et al.* as it produces lesions in infant mice in

skeletal muscles and viscera, with little evidence of affection of the central nervous sytem. This grouping can, however, be only very tentative, because of the relatively small number of animals so far examined, the possible differences in susceptibility of the tissues of different strains of mice, as Pappenheimer *et al.* point out, and further because of the definite time interval which elapses after inoculation before a visible lesion appears. This latter aspect was studied by Melnick and Godman (1951) for the Conn 5 strain. The present study also indicates that in infant mice lesions in the fat pads, liver and pancreas appear relatively early (third day), muscle lesions a little later (fourth day). Animals dying on the second day after receiving a large dose of virus show little histological change except a mild diffuse hepatic necrosis.

In contrast to other work on Coxsackie viruses, we have found that adult mice may be infected with our virus and show an intense pancreatitis without definite evidence of specific lesions in other organs.

SUMMARY.

A virus pathogenic for infant mice has been isolated from pharyngeal washings of a case of pleurodynia. It produces retardation of growth, and focal and diffuse inflammatory lesions in skeletal muscle, fat pads, liver and pancreas.

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ADDENDUM.

While this paper was going to press Pappenheimer, *et al.* (J. exp. Med. 1951, 94, p. 45) described pancreatitis in adult mice infected with the Conn. 5 strain of Coxsackie virus.

THE RÔLE OF THE ADRENAL CORTEX IN DIABETIC KETOSIS

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Many non-specific stress states are associated with an increase in adrenocortical activity. Diabetic ketosis produces a severe disturbance in fluid, electrolyte, carbohydrate, protein and fat metabolism and it would seem most likely that these disturbances would throw a considerable stress on the whole organism. These experiments were designed to evaluate the level of adrenocortical activity in diabetic ketosis. The investigations have an added significance because certain of the adrenal steroids—particularly the 11, 17 oxysteroids—have a profound effect on the diabetic state.

It has been suggested that the aggravation of diabetes with infections, fractures and other types of stress, is due to an increase in adrenal activity with consequent overproduction of carbohydrate active corticoids (Albright, 1943; Shipley *et al.*, 1946; Selye, H., 1946). Trauma and other stresses in normal man and animals are often followed by a glycosuria and reduced tolerance to glucose (Kelley and McDonald, 1948; Stickney *et al.*, 1948; Thomsen, 1938). Even psychic stress has been shown to cause a rise in blood level in normal, but more especially in diabetic, patients. The rise in blood ketones was proved by hepatic vein analysis to be due to excessive ketone production (Hinkle *et al.*, 1950).

The diabetogenic and ketogenic effects of A.C.T.H. and 11 oxysteroids have been adequately demonstrated both by subtraction and addition experiments.

Hypophysectomy ameliorates the diabetic state and produces insulin sensitivity in normal animals (Houssay and Potick, 1929). Anterior pituitary extract (A.P.E.) prevents the insulin sensitivity (Houssay and Potick, 1929; Young, 1938), and part of this action is due to A.C.T.H. Administration of purified A.C.T.H. causes excessive ketonuria of fasted rat (Bennett *et al.*, 1948a) and normal dog (Bennett *et al.*, 1948b), and enhances diabetes only if the adrenal cortex is present (Long, 1937a and b). A.C.T.H. can also produce hyperglycaemia, glycosuria and diabetic glucose tolerance curves in normal men and animals (Ingle *et al.*, 1946; Conn *et al.*, 1948; Forsham *et al.*, 1948; Conn, 1950). Moreover, A.C.T.H. causes a state of relative insulin resistance in normal mice (Grattan and Jensen, 1940), humans (Conn, 1950) and diabetic rats (Bennett and Li, 1947). Long (1940) has recently reviewed the rôle of adrenal cortex in carbohydrate, protein and fat metabolism. Adrenalectomy produces insulin sensitivity (Lewis *et al.*, 1940) and improves the diabetic state almost as dramatically as hypophysectomy (Long *et al.*, 1940; Ingle and Thorn, 1941; Ingle and Prestrud, 1948). The low blood sugar, hypoglycaemic attacks and decreased hyperglycaemic response to adrenaline of Addison's disease can all be treated with compound E, but not with D.O.C.A. (Thorn *et al.*, 1940). Adrenal hormones, especially the 11, 17 oxysteroids, can aggra-

vate the diabetic state, (Long *et al.*, 1940; Ingle, 1944), produce ketonuria, and create a marked insulin resistance. Normal force-fed rats given 5 mg. of compound E each day exhibited a glycosuria which could not be controlled by 100 units of P.Z. insulin/day (Ingle, 1941).

It therefore seems likely that if there is an increase in adrenal cortical activity during diabetic ketosis a vicious circle would be set up. The increased A.C.T.H. and corticosteroid secretion would aggravate the diabetic state, increase the ketosis, glycosuria, nitrogen excretion and establish a state of relative insulin resistance. This might be at least part of the explanation why patients in a diabetic ketotic coma require such huge doses of insulin.

Method.

Adult male white, hooded and black rats weighing between 300–320 gm. and bred at the Baker Medical Research Institute were used in these experiments. The animals were made diabetic by the intravenous administration of alloxan at a dose level of 50 mg./kg. They were then placed in individual metabolism cages and fed on a relatively constant diet of cabbage leaves, meat, specially prepared cubes and water *ad libitum*. (In addition each animal received by subcutaneous injection 0·2 ml. of peanut oil per day, so that they could be used as controls for another experiment.) All animals were tested for diabetes by determination of blood sugar (Hagedorn and Jensen, 1923). Qualitative tests for glycosuria and urinary ketones were also carried out. One group of diabetic rats was given no special treatment. These developed glycosuria, marked ketosis and blood sugars of 300–600 mg. p.c. The other diabetic group was controlled as well with P.Z. insulin. The insulin injections were commenced 24 hours after alloxan administration and 3–6 units P.Z. insulin were given daily according to urinary findings. These rats did not develop ketosis, although some did show a moderate to severe glycosuria. Blood sugar ranged from 120–300 mg. p.c.

Ninety-six hours after alloxan administration, the animals were killed by a blow on the head. The adrenals and spleen were immediately removed. The adrenals were placed on filter paper and carefully dissected free from all peri-adrenal connective tissue and fat, weighed and then placed into 6 p.c. trichloracetic acid if ascorbic acid estimation was required, or into alcohol-ether if adrenal cholesterol was to be measured. Adrenal ascorbic acid was measured by the method of Roe and Kuether (1943), and cholesterol by the method of Sackett (1925) modified for tissues. The spleens were dissected free from connective tissue, placed into individual small glass vials and dried in an oven at 80° C. for 12 hours. They were then allowed to cool in a desiccator to room temperature before being weighed.

RESULTS.

Three groups of rats were compared:

1. Normal controls. 2. Uncontrolled alloxan diabetic ketotic. 3. Alloxan diabetic controlled with P.Z. insulin.

Table 1 shows the results of wet adrenal weights, adrenal ascorbic acid concentrations, adrenal cholesterol concentrations and dry splenic weights in each of these groups.

Fig. 1 summarizes these results and also indicates the comparisons which are statistically significant.

All rats made diabetic had heavier adrenals. This was particularly so in that group which did not receive insulin and hence became ketotic. Some of the adrenals of this latter group showed haemorrhagic areas and were red-brown in colour instead of yellow. These changes are due to loss of cytoplasmic lipid and constitute evidence of severe adrenal hyperactivity or even exhaustion (Sayers *et al.*, 1944).

TABLE 1.

		Adrenal weight (mg.)	Adrenal ascorbic acid concentration (mg./100 gm. adrenal)	Adrenal cholesterol gm./100 gm. adrenal)	Dry splenic weight (mg.)
Normal	No. of observations:	12	12	12	12
	Mean:	39·6	385·3	4·62	266
	Standard deviation:	3·3	45	0·36	44
	Range:	36·9–48·3	314–478	4·06–5·15	211–394
Alloxan diabetic insulin controlled		12	12	12	12
		50·3	405·3	3·66	236
		2·8	68	1·07	81
		46·4–55·1	307–536	1·82–5·12	115–336
Alloxan diabetic ketotic		24	18	18	12
		60·0	318·3	0·84	100
		4·5	105	0·35	25
		52·7–70·8	154–458	0·39–1·62	70–141

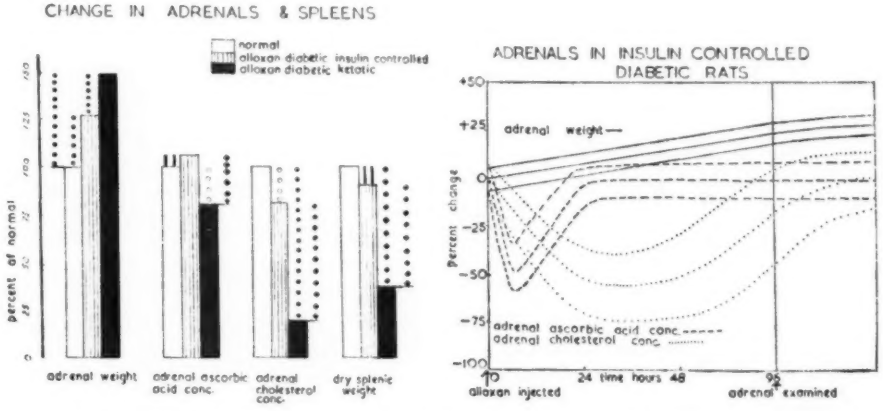

Fig. 1. Graphic comparison of adrenals and spleens in the three groups of experimental animals.

●● represents a difference which is statistically significant at the 0·1 p.c. level; ◆◆ at the 1 p.c. level; and OOO at the 5 p.c. level. ▌▌ represents a difference which is not statistically significant.

Fig. 2. Probable theoretical curves of changes with respect to time of adrenal weight, cholesterol and ascorbic acid concentration following the administration of alloxan. The diabetes was controlled with insulin. The adrenals are examined 96 hrs. after alloxan administration. The adrenals are 25 p.c. heavier, the depleted ascorbic acid values have returned to normal and some but not all the cholesterol values have returned to normal. Therefore the mean cholesterol concentration is lower than normal and is made up of a fairly wide scatter.

The mean adrenal ascorbic acid concentration of the insulin-treated diabetic group did not differ from normal. Those animals allowed to become ketotic had a significantly low mean ascorbic acid and moreover this mean was found to be made up of values ranging from very low to normal. Apparently some animals who are ketotic and have enlarged adrenals show a normal ascorbic acid value. The particular significance of this result will be discussed later. There was an extreme and consistent fall in adrenal cholesterol in the diabetic ketotic animals. The insulin-treated animals have a mean cholesterol value below normal and this mean is made up of values ranging from very low to normal. The range of values for ascorbic acid is wide in the diabetic ketotic group whereas the cholesterol values show a greater scatter in the insulin-treated non-ketotic group. The results for the dry splenic weights follow the same pattern as the cholesterol values. The spleens weigh much less in the diabetic ketotic than normal, whereas the insulin-treated group have a mean splenic weight only a little below normal.

DISCUSSION.

Increase in adrenal weight occurs within 6–24 hours after the application of a wide variety of stress states, and A.C.T.H. administration (Li *et al.*, 1943; Sayers *et al.*, 1943). The wet or dry adrenal weight is a good index of activity in experiments of more than a few days' duration.

Increase in adrenal activity is associated with a decrease in adrenal cholesterol and ascorbic acid concentration (Sayers et al., 1948). Following a single short stimulus, or after a prolonged stimulus (provided this latter is not extremely severe), the depleted adrenal ascorbic acid and cholesterol values tend to return to normal. In the rat adrenal the ascorbic acid becomes depleted and recovers more rapidly than does the cholesterol. The biphasic nature of each of these adrenal responses and the fact that the time intensity relationship of one differs from that of the other, is probably the reason why the adrenal ascorbic acid-cholesterol values do not always run together. Other investigators have noticed this lack of correlation between the ascorbic acid and cholesterol findings under various circumstances (Pinchot *et al.*, 1949; Tepperman *et al.*, 1947). Moreover, if the adrenal is examined at that particular time when the depleted values are just recovering, one would expect to find a wider scatter of results with a mean below normal. The application of these principles in the interpretation of the results obtained in this experiment are represented in Figs. 2 and 3.

The alloxan diabetic insulin-treated non-ketotic group of rats shows evidence of increased adrenal cortical activity. The adrenals are heavier, the mean adrenal ascorbic acid concentration is normal, but the slower changing cholesterols have not yet all returned to their original pre-stress values and so their mean is somewhat below normal. Apparently 96 hours after alloxan administration the cholesterol concentration has returned to normal in some adrenals but not in others. It is evident that the adrenal ascorbic acid by itself is not a reliable index of the functional state of the adrenal which has been subjected to prolonged stress (Pinchot *et al.*, 1949). The stress in this particular experiment is probably due to the alloxan administration *per se* and also the controlled diabetic non-ketotic state. Alloxan has been shown to produce changes in the adrenal. The adrenal is usually described as normal or only having minor changes, though necrosis, congestion and haemorrhages have occasionally been seen (Greep and Deane, 1949). It is believed that the adrenal

changes are mostly caused by the diabetic state rather than by the transient toxic effects of the alloxan (Bennett and Koneff, 1946). Deane *et al.* have demonstrated that experimental conditions associated with a modified output of carbohydrate-regulating hormones produce histological and histochemical lipoid changes in the zona fasciculata but not in the zona glomerulosa (Deane and Greep, 1946; Deane *et al.*, 1948). Since the changes described in the rat adrenal cortex after alloxan administration are confined to the zona fasciculata it seems likely that the carbohydrate regulatory hormones of the 11, 17 oxysteroid type are involved (Applegarth, 1949).

The untreated alloxan diabetic ketotic animals have been subjected to a greater and more prolonged stress as evidenced by the gross increase in the adrenal weight. One would expect the ascorbic acid and cholesterol curves to be shifted towards the right as seen in Fig. 3. Therefore when the adrenals are examined 96 hours after alloxan administration one finds a consistent and profound fall in cholesterol associated with a series of widely scattered ascorbic acid values. Untreated diabetes, especially when associated with ketosis, produces a severe prolonged stimulation of the adrenal cortex amounting almost to exhaustion.

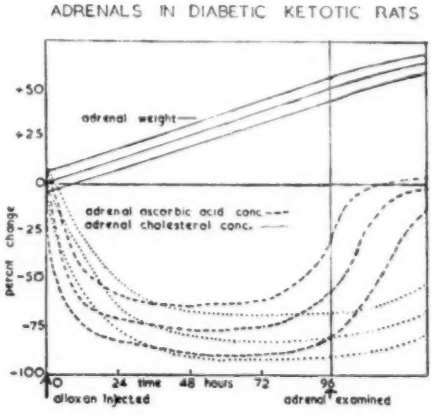

Fig. 3. Probable theoretical curves of changes with respect to time of adrenal weight, cholesterol and ascorbic acid concentration following the administration of alloxan. The animals were not given insulin and therefore developed ketosis. The adrenals are examined 96 hours after alloxan administration. The adrenals are 50 p.c. heavier, there is a profound and consistent depletion of cholesterol and some but not all ascorbic acid values have returned to normal.

In experimental animals the total mass of lymphoid tissue varies inversely with the activity of the adrenal cortex (Dougherty and White, 1943). However, other factors such as fasting and gluconeogenesis from proteins during diabetic ketosis could influence the lymphoid tissue mass. In these experiments the dried splenic weight has been used as an index of total lymphoid tissue. The results confirm the adrenal findings. It is interesting to note (Fig. 1) that the splenic weight values follow fairly accurately the same pattern as adrenal cholesterol.

Examination of adrenals gives an indication as to the level of adrenocortical activity. Sayers and Sayers (1947, 1948) suggest that there is an increased requirement and utilization of corticosteroids during stress. Therefore increased adrenocortical activity as evidenced by adrenal changes may be accompanied by even relative deficiency of corticosteroids as far as the metabolic requirements of the tissue cells are concerned. One must look for change in the organism for the answer to this problem. In these experiments the lymphoid tissue weight

has decreased. But since the weight of lymphoid tissue would also be influenced by the protein catabolic and gluconeogenic stress of uncontrolled diabetes, this index as a level of adrenocortical activity may be invalid. Therefore we have no direct proof from these experiments that in diabetic ketosis the tissue cells are being flooded with excess corticosteroids even though we do know that there is an increased adrenocortical activity.

Trauma in man and animals is often followed by glycosuria and reduced glucose tolerance (Kelley and McDonald, 1948; Stickney *et al.*, 1948; Thomsen, 1938). Moreover, trauma, infections and other stresses can aggravate a diabetic state (Albright, 1943; Shipley *et al.*, 1946; Kelley and McDonald, 1948). The inference from these observations would seem to be that the aggravation of the diabetes is due to hyperactivity of the adrenal cortex with consequent overproduction of carbohydrate-active corticosteroids. However, adrenalectomised alloxan diabetic mice receiving a fixed daily amount of adrenal cortical extract exhibit as great a rise in fasting blood sugar after typhoid injections as do intact alloxan diabetic mice (Tobian and Strauss, 1949). The hyperglycaemic reaction to trauma fails to occur in the absence of the adrenals. But a fixed daily dose of adrenal cortical extract which by itself does not elevate the blood sugar of non-traumatized rats restores the hyperglycaemia of trauma in adrenalectomized animals (Selye and Dosne, 1941). Similarly, adrenalectomised animals maintained on a constant daily dose of adrenal cortical extract (which by itself did not cause an increase in nitrogen excretion) exhibited as great an increase in urinary nitrogen excretion following fracture as did normal animals (Ingle *et al.*, 1947). These observations indicate that the adrenal plays a supportive rather than a causal rôle in the hyperglycaemia, decreased glucose tolerance and increased nitrogen excretion of stress. Therefore, even though diabetic ketosis is associated with an increased adrenocortical activity it does not logically follow that the aggravation of diabetes which occurs during ketosis is necessarily due to adrenal overactivity.

SUMMARY.

There is a moderate increase in adrenocortical activity in insulin controlled alloxan diabetic rats.

There is a very marked increase in adrenocortical activity in uncontrolled alloxan diabetic ketotic rats.

The insulin resistance and aggravation of diabetes which occur during ketosis are probably related to the increased adrenocortical activity and consequent over-production of 11, 17 oxysteroids. The validity of this causal relationship has been discussed.

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